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HUMAN RENAL TRANSPLANTATION: AN INVESTIGATION OF THE FUNCTIONAL STATUS OF THE DENERVATED KIDNEY AFTER SUCCESSFUL HOMOTRANSPLANTATION IN IDENTICAL TWINS

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In a study of renal function in a patient with a successfully homotransplanted kidney, Bricker and co-workers[2] observed normal function except for abnormal sodium excretion after acute alterations of extracellular fluid volume (ECFV). The present studies were performed after successful homotransplantation in which the transplanted kidney was ischemic for a shorter period and bilateral nephrectomy was performed earlier than in the previous study.

Effective renal plasma flow and glomerular filtration rate were within normal limits for a single kidney. The ability to acidify and alkalinize urine, and to concentrate and dilute urine were normal. The kidney displayed normal diurnal variation in water and sodium excretion. When orthostatic hypotension was induced, there was slight decrease in inulin and para-aminohippurate clearance. The responses to acute changes in ECFV were normal. During rapid infusion of hypotonic saline sodium excretion, free water clearance and osmolar clearance increased. After isotonic saline infusion in the hydropenic state, there was increase in sodium excretion and osmolar clearance with elaboration of hypertonic urine. Elaboration of hypertonic urine with increase in free water clearance and osmolar clearance followed rapid infusion of isotonic saline in the prehydrated state. During the rapid infusion of hyperoncotic salt poor albumin osmotic urine to plasma ratios increased with decrease in water and solute excretion. It is concluded that renal nerves are not necessary for the integrity of renal function in the diversified parameters studied.

THE opportunity to study the functional status of the denervated human kidney presented itself after successful homotransplantation in an identical twin.[1] Bricker and associates[2] have reported that the transplanted human

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TABLE I. REPRESENTATIVE LABORATORY DATA REVEALING PROGRESSIVE COURSE OF DISEASE AND BENEFICIAL EFFECT OF RENAL TRANSPLANT

DATE	SP. GR.	W.B.C.	R.B.C.	CASTS	ALBUMIN	PSP %	HGB.	TSP	NPN	BUN	Na	K	Cl	CO_2	Ca	P	Cr
6-17-53	1.025	Rare	Occ	Occ	1+	70	14.9		35		144	4.3	113	21			1.2
8-23-56	1.020	Occ	1-5	6-8	3+	43	14.3		33		144	4.3	113	21			1.2
10-28-57	1.009	8-9	9-10	8-10	4+	10	11.7	5.8	51	30	136	5.4	100	20	5.1	5.7	2.1
12- 2-57	1.008	15-20	9-10	Inn	4+	0	11.		126	108	134	5.0	99	19	4.6	7.0	10.
12-10-57	1.007	15-20	9-10	Inn	4+		8.2	6.2		124	136	5.6	94	17			16.2
Hemodialysis was performed on 12-10-57.																	
12-11-57	1.008	15-20	9-10	Inn	4+		11			54	144	4.4	97	26	5.	5.2	10.
Renal transplant procedure was performed on 12-12-57																	
12-15-57	1.011						11.6			27	134	3.4	95	26			2.8
12-25-57	1.019	Rare	Rare	Rare	1+	27	10.6	6.2		20	139	5.5	103	20			1.0
Bilateral nephrectomy was performed on 1-3-57. The results below were on the transplanted kidney.																	
1- 5-58	1.009	8-16	Rare	0	1+		11			35	132	5.4	102	22			2.8
1-14-58	1.014	8-10	0	0	1+		12.6			21	135	4.2	99	22			2.2
2-13-58	1.019	0	0	0	0	72	12.8			23							1.4
3- 5-58	1.022	0	0	0	0		13.2	6.2		21	142	5.0	106	23	4.9	4.1	1.4
1- 4-59	1.028	0	0	0	0		14.5	6.8		20	143	4.7	103	24			1.1

PSP = phenolsulfonphthalein; TSP = total serum protein; NPN = nonprotein nitrogen; BUN = blood urea nitrogen.

kidney behaved in a normal manner except for abnormalities in sodium excretion after acute extracellular fluid volume alterations. They suggested that this was probably because denervation interrupted the efferent limb of a volume control system. The transplanted kidney was ischemic for 89 minutes and there was a 5 months' lapse between transplantation of the kidney and surgical removal of the diseased organs. These factors may have contributed to the abnormalities they noted. Since the patient who is the subject of this report differed in having only a 47 minute period of ischemia and a shorter period elapsed (3 weeks) before nephrectomies, further study of the problem seemed indicated.

Case Report.—The recipient (R) was in good health until early 1953 when he developed frequent headaches and easy fatigability. In May, 1953, he was found to have hematuria, 1 to 2 plus proteinuria, and a blood pressure of 140/90 mm. Hg. A diagnosis of chronic glomerulonephritis was made. Intravenous pyelogram, phenolsulfonphthalein excretion, nonprotein nitrogen, electrocardiogram, and chest x-ray were within normal limits. Except for mild headaches and occasional ankle and periorbital edema he was relatively asymptomatic until late September, 1957, when he developed severe persistent headaches and noted blurring of vision and periorbital and pedal edema. His physician* noted a blood pressure of 200/130 mm. Hg, grade IV fundi, and 4 plus proteinuria, and referred him to the hospital.

The physical examination on admission revealed a pallid, afebrile, thin, slightly lethargic, 26-year-old white man who appeared chronically ill. Blood pressure was 240/140 mm. Hg, pulse rate was 110 per minute, and respirations were 24 per minute. The fundi were grade IV. The heart was not enlarged. A high pitched systolic murmur was heard over the apex. The second pulmonic sound was moderately increased. No abdominal masses were palpated. There was mild bilateral costovertebral angle tenderness. There was 1 plus pedal and pretibial edema. Neurologic examination was within normal limits.

Representative laboratory data are presented in Table I. Urine cultures were negative; urine protein excretion was 10.8 Gm. per 24 hours; antistreptolysin-O titer was 50 units; and C-reactive protein was negative. The electrocardiogram was normal on admission but by November, 1957, the intrinsicoid deflection was prolonged (0.05 second) in lead V_4 suggesting left ventricular hypertrophy.

During the 2 months after admission the patient deteriorated markedly. The patient had a twin brother, thought to be identical, and renal homotransplantation was considered. Physical examination and laboratory studies on the donor twin (D) revealed that he was in excellent health. Blood types on the twins† were identical. Split and full thickness skin grafts were exchanged between twins with no evidence of local rejection.

*We wish to thank Dr. William Rucks, Oklahoma City, Okla., for referring this patient.

†Performed in the hematology laboratories of Dr. Robert Bird, University of Oklahoma Hospital, Oklahoma City, Okla., and Dr. Phillip Levine, Ortho Laboratories, Raritan, N. J.

The twins were transported to the Medical College of Virginia, Richmond, Virginia, and on Dec. 12, 1957, 2 days after extracorporeal hemodialysis, homotransplantation was performed. The normal left kidney of D was transplanted to the right iliac fossa of R, anastomosing the renal artery end-to-end with the hypogastric artery, the renal vein end-to-side with the common iliac vein, and the ureter mucosa-to-mucosa with the urinary bladder. The total ischemic period of the transplanted organ was 47 minutes. There was prompt urine excretion in copious amounts after the anastomoses were completed. D's postoperative course was uneventful. R's condition improved rapidly and on the twenty-first posttransplantation day his diseased kidneys were removed in a 1 stage procedure. After this his blood pressure stabilized around 120/70 mm. Hg and he gained 32 pounds in weight over the following year. His electrocardiogram returned to within normal limits. Numerous 24 hour urine protein determinations have revealed no protein. Hemograms and blood chemistries (as depicted in Table I) have remained within normal ranges.

PROCEDURE OF INVESTIGATION

Control clearances on D were obtained prior to transplantation for a 2 kidney control and 1 month after his left nephrectomy for a 1 innervated kidney control. The majority of studies on R were made over a 9 month period beginning 2½ months after his 1 stage bilateral nephrectomy.

The studies were performed in the morning with the subjects postprandial and recumbent, unless otherwise indicated by specific tests. Voided specimens rather than catheterized specimens were used in all the studies and recorded values of inulin and para-aminohippurate (PAH) clearance are averages of three clearance periods. Effective renal plasma flow (ERPF) was determined by the clearance of PAH (C_{PAH}), glomerular filtration rate (GFR) by the clearances of inulin (C_{In}), and trends in the glomerular filtration rate were followed by the clearance of endogenous creatinine (C_{Cr}). PAH was analyzed utilizing the method of Smith and collaborators,[3] inulin according to the method of Roe and co-workers,[4] and endogenous creatinine as measured by the method of Bonsnes and Taussky.[5]

Chloride was determined by the method of Van Slyke and Hiller[6]; sodium and potassium were analyzed on an internally compensated Barclay flame photometer; urine and plasma osmolalities were determined on a Fiske Osmometer; pH was measured on a Beckman pH meter; and urine specific gravity was measured on an E. H. Sargent and Company urinometer at 77° F.

(For purposes of correcting the clearance values to standard body surface, R's body surface area was 1.66 sq.M. in all but the 12 month follow-up when it was 1.70 sq.M., and D's surface area was 1.70 sq.M. The recorded values have not been corrected to standard body surface area.)

RESULTS

Glomerular Filtration Rate and Renal Plasma Flow.—(Table II.) Two kidney pretransplantation control studies on D revealed C_{In} of 118 ml. per minute and C_{PAH} of 587 ml. per minute. One month after his left nephrectomy C_{In} was 66 ml. per minute and C_{PAH} 440 ml. per minute.

C_{In} on R after transplantation but prior to bilateral nephrectomy was 38 ml. per minute. One month after bilateral nephrectomy C_{In} was 42 ml. per minute and thereafter increased to 60 ml. per minute where it has remained. R's C_{PAH} was 239 ml. per minute after transplantation but prior to bilateral

TABLE II. MEASUREMENTS OF C_{In}, C_{PAH}, AND C_{Cr}

	MO. AFTER TRANSPLANT	C_{Cr} (ML./MIN.)	C_{In} (ML./MIN.)	C_{PAH} (ML./MIN.)	FILTRATION FRACTION (%)
Donor*: 2 kidney control			118	587	20.1
Donor*: After removal of left kidney	1		66	440	12.8
Recipient†: Before bilateral nephrectomies	½		38	239	16.3
Recipient†: After bilateral nephrectomies	¾				
	1	78	42	340	12.4
	1½	74.1			
	1¾	73.4			
	2	74.0	60.6	353.8	17.1
	2½	71.2	58.0	373.1	15.5
	2½	68.0	59.1	379.2	15.5
	3	67.3	62.3	408.9	15.2
	3¼	72.6	59.2	372.0	15.9
	4	75.3			
	12		60.7	387.5	16.1

*Surface area = 1.70 sq. M.
†Surface area = 1.66 sq. M.

TABLE III. ACIDIFICATION AND ALKALINIZATION OF URINE

SPECIMEN	VOLUME (ML./MIN.)	pH	TITRATABLE ACIDITY (μEq/MIN.)	$U_{Na}V$ (μEq/MIN.)	U_KV (μEq/MIN.)	$U_{Cl}V$ (μEq/MIN.)
−2	0.82	5.04	16.4	85.8	53.7	120.3
−1	0.85	5.01	17.0	93.9	49.4	124.4
1	2.40	5.21		176.4	73.4	229.2
2	6.67	6.11		260.1	100.0	333.5
3	6.33	6.27		179.1	93.1	253.2
4	5.76	6.51		141.1	102.5	220.0
5	5.50	6.31		118.3	96.3	195.3
6	5.47	6.20		108.3	102.3	181.6

One gram ammonium chloride was given every 4 hours over a 16 hour period prior to the test. After a control of two 30 minute collections, the patient was given 250 mg. Diamox "O" at 0 time and additional 30 minute specimens were taken.

TABLE IV. DIURNAL VARIATION IN WATER AND ELECTROLYTE EXCRETION

TIME	EOS. COUNT	VOLUME (ML./MIN.)	$U_{Na}V$ (μEq/MIN.)	U_KV (μEq/MIN.)	$U_{Cl}V$ (μEq/MIN.)
8:00 A.M.	223	1.13	142.4	34.2	117.5
11:00 A.M.	212	1.21	130.7	62.7	200.0
2:00 P.M.	215	1.20	138.0	71.5	151.0
5:00 P.M.	198	1.08	112.3	46.9	114.5
8:00 P.M.	184	.66	56.1	15.0	64.7
11:00 P.M.	190	.89	96.1	23.0	84.5
2:00 A.M.	286	.97	44.6	15.9	89.2
5:00 A.M.	171	1.11	56.1	27.4	133.2
8:00 A.M.	209	1.16	146.2	38.7	143.8

Over a 24 hour period R was given 250 ml. water each 3 hours. Eosinophil count, urine volume, urine sodium, potassium, and chloride were determined each 3 hours.

TABLE V. RESPONSE TO HYPOTONIC EXPANSION OF ECFV

TIME	VOLUME	C_{Cr}	OSM (U/P)	C_{Osm}	C_{H_2O}	$U_{Na}V$ (μEq/MIN.)	U_KV (μEq/MIN.)	$U_{Cl}V$ (μEq/MIN.)	$U_{Osm}V$	P_{Na}	P_k	P_{Cl}	TSP	Hct
−105 to −75	4.1	71	0.59	2.4	1.7	166	75	166	922	145	5.5	106	6.5	42
−75 to −45	4.6	69	0.46	2.1	2.5	129	69	138	626					
−45 to −10	8.2	75	0.44	3.6	4.6	248	106	229	1,046	140	4.1	105	6.5	40
−10 to 15	6.3	74	0.44	2.8	3.6	232	88	189	808					
15 to 40	6.9	71	0.42	2.9	4.0	242	103	206	846					
40 to 65	8.6	73	0.42	3.6	5.0	336	128	325	1,038	134	3.9	107	5.5	37
65 to 90	7.9	68	0.41	3.3	4.6	332	103	317	952					
90 to 115	8.3	72	0.42	3.5	4.8	419	117	300	998					
115 to 140	7.8	70	0.41	3.2	4.6	314	94	282	933	137	3.9	108	6.2	36
140 to 165	7.2	72	0.39	2.8	4.4	277	86	202	814					
165 to 190	7.8	70	0.39	3.0	4.8	358	55	220	878					
190 to 215	8.6	73	0.38	3.2	5.4	349	69	257	933	137	4.1	106	6.3	38

In the prehydrated state the patient was given 2,000 ml. of hypotonic solution (126 mEq. Na, 102 mEq. Cl, 24 mEq. HCO_3) intravenously beginning at 0 time over a 65 minute period.

nephrectomy and 354 ml. per minute 2 months after 1 stage bilateral nephrectomy. Thereafter C_{PAH} has varied without apparent trend but has ranged above 370 ml. per minute. Filtration fractions have varied from 15.2 to 17.1 per cent, again without apparent trend. The composite mean value of C_{Cr}/C_{In} was 1.21.

TABLE VI. RESPONSE TO ISOTONIC SALINE IN HYDROPENIC STATE

TIME	VOLUME (ML./MIN.)	C_{Cr}	$U_{Na}V$ (μEq/MIN.)	U_KV (μEq/MIN.)	$U_{Cl}V$ (μEq/MIN.)	$U_{Osm}V$	OSM U/P	C_{Osm}	C_{H_2O}
-31 to 14	0.9	66.0	112	48	135	661	2.34	2.18	-1.25
14 to 55	1.4	65.6	191	59	215	843	2.03	2.77	-1.41
55 to 85	1.5	62.6	231	58	272	871	1.88	2.88	-1.35
85 to 107	2.5	61.0	375	72	408	1,273	1.88	2.88	-1.35
107 to 140	2.7	61.3	368	75	404	1,131	1.52	3.88	-1.33
140 to 158	2.7	62.6	419	93	452	1,301	1.61	4.28	-1.62
158 to 178	2.5	61.1	398	95	425	1,223	1.61	4.03	-1.53

After a 15 hour NPO period the patient was given 3,000 ml. of normal saline intravenously over a 70 minute period beginning at 0 time.

Acidification and Alkalinization.—(Table III.) The recipient was given 1.0 Gm. of nonenteric coated ammonium chloride every 4 hours for 16 hours prior to the study. The following morning voided urine specimens were collected under oil at 30 minute intervals. After 2 control samples 250 mg. of Diamox (acetazolcamide) was given by mouth and 6 further samples were taken.

After the administration of Diamox the urine pH increased from a control of 5.01 to 6.51. Titratable acidity decreased from 17 μEq per minute to zero in less than 30 minutes and excretion rates of sodium, potassium, and chloride increased significantly. Urinary output increased from 0.82 ml. per minute to 6.67 ml. per minute.

TABLE VII. RESPONSE TO ISOTONIC SALINE IN PREHYDRATED STATE

TIME	VOLUME (ML./MIN.)	C_{Cr}	$U_{Na}V$ (μEq/MIN.)	U_KV (μEq/MIN.)	$U_{Cl}V$ (μEq/MIN.)	$U_{Osm}V$	OSM (U/P)	C_{Osm}	C_{H_2O}
-14 to 20	1.1	59.8	127	36	144	512	1.61	1.71	-0.65
20 to 42	2.6	65.3	217	57	238	764	0.99	2.56	0.03
42 to 69	5.7	65.4	378	86	384	1,045	0.61	3.51	2.23
69 to 97	8.3	67.4	508	99	511	1,353	0.55	4.54	3.71
97 to 118	6.2	72.6	557	105	581	1,498	0.81	5.03	1.16
118 to 140	3.3	66.2	516	83	546	1,000	1.00	3.31	0.00
140 to 168	2.8	65.1	451	85	490	953	1.12	3.16	-0.34

Two thousand milliliters of water was given "o" between -12 and -11 hours. Beginning at 0 time 3,000 ml. of normal saline was given intravenously over a 70 minute period.

Concentration and Dilution.—Diluting capacity of R was determined by his ability to elaborate a hypotonic urine after acute water loading. The minimal specific gravity obtained was 1.001 and the minimal osmotic urine to plasma (U : P Osm.) ratio was 0.32. Concentrating ability was evaluated after 14 hours of water deprivation. The maximal specific gravity was 1.030 and the maximal osmotic urine to plasma ratio was 2.96.

TABLE VIII. EFFECTS OF RAPID INFUSION OF HYPERONCOTIC SALT POOR HUMAN ALBUMIN

PATIENT	TIME (MIN.)	C_{Cr} (ML./MIN.)	VOLUME (ML./MIN.)	C_{Osm} (ML./MIN.)	C_{H_2O} (ML./MIN.)	OSM (U/P)	$U_{Na}V$ (μEq/MIN.)	$U_{Cl}V$ (μEq/MIN.)	$U_{K}V$ (μEq/MIN.)	P_{Na} (mEq./L.)	P_{Cl} (mEq./L.)	P_{K} (mEq./L.)
1,500 ml. water ingested from –110 to –65												
1	–120 to –90	100	0.9	2.3	–1.4	2.1	130	146	26	144	109	4.4
2	– 90 to –60	91	2.0	3.0	–1.0	1.5	134	126	26	143	107	4.4
3	– 60 to –40	98	6.2	5.1	1.1	0.82	127	130	25			
4	– 40 to –25	101	6.8	2.9	3.9	0.42				143	106	4.2
5	– 25 to – 5	103	7.2	2.9	4.3	0.40	129	127	26	142	107	4.3
250 ml. 25 per cent salt poor human albumin infused from 0 to 45 minutes												
6	– 5 to 34	90	5.5	2.4	3.1	0.44	79	111	27	137	106	4.5
7	34 to 60	98	5.7	2.6	3.1	0.46	67	105	28	138	105	4.3
8	60 to 93	93	6.4	2.8	3.6	0.44	81	111	30	139	106	4.2
9	93 to 121	96	7.5	3.1	4.4	0.41	79	122	27	139	107	4.2
10	121 to 150	91	7.7	3.2	4.5	0.42	92	125	27	140	105	4.3

Diurnal Variation.—(Table IV.) Over a 24 hour period R was given 250 ml. of water by mouth each 3 hours. Eosinophil counts were determined each 3 hours and urine was collected each 3 hours and volume, sodium excretion per minute ($U_{Na}V$), potassium excretion per minute (U_KV), and chloride excretion per minute ($U_{Cl}V$) were determined. During the night hours there was a decrease in urine volume, sodium output, potassium output, and chloride output, as is usually found in normal individuals.[7]

Effect of Changing of Body Position.—(Fig. 1.) In order to determine whether alterations in body position would significantly effect GFR, ERPF, and electrolyte excretion, C_{PAH}, C_{In}, C_{Cr}, $U_{Na}V$, U_KV, $U_{Cl}V$, blood pressure, and pulse were measured first during control periods with the patient in a supine position, during a period of quiet sitting, during a period of quiet standing, and during a follow-up period in the supine position. Urine minute volume decreased during quiet standing. There were transient mild decreases in blood pressure on sitting and on standing, but the blood pressure rapidly returned to near control levels. There was no significant change in C_{PAH} on sitting and a moderate decrease on standing. C_{In} remained stable on sitting and decreased slightly on standing and C_{Cr} corresponded with C_{In}. Electrolyte excretion diminished as the filtration fraction increased during the standing period and then returned toward control levels.

Effect of Changes in Extracellular Fluid Volume.—

Infusion of hypotonic saline: (Table V). The patient (R) was studied by the method suggested by Strauss and collaborators.[10] He was given 2,000 ml. of hypotonic solution (126 mEq. Na, 102 mEq. Cl, 24 μEq HCO_3) intravenously over a 65 minute period. C_{Cr} remained fairly constant. Urine flow increased from 4.1 ml. per minute to 8.6 ml. per minute. $U_{Na}V$ increased from 129 to 419 μEq per minute and $U_{Cl}V$ from 138 to 325 μEq per minute; free water clearance (C_{H_2O}) increased from 1.7 to 5 ml. per minute whereas osmolar clearance ($C_{Osm.}$) increased slightly. The hypertonic state of the patient is evidenced by the decrease in serum sodium and potassium, in serum protein, in hematocrit, and in plasma osmolality (from 295 to 289 mOsm.).

Infusion of isotonic saline, hydropenic state: (Table VI.) The recipient was given 3,000 ml. of isotonic saline intravenously over an 80 minute period after a 15 hour fasting period to test his response to the hydropenic state. There was a decrease in C_{H_2O} (–1.25 to –1.62), an increase in urine sodium excretion (113 to 419 μEq per minute), an increase in C_{Osm} (2.18 to 4.28 ml. per minute), and an elaboration of a hypertonic urine with a minimal osmolar U : P of 1.40.

Infusion of isotonic saline, prehydrated state: (Table VII.) R was given 3,000 ml. of isotonic saline intravenously over a 70 minute period after having been given 2,000 ml. of water by mouth 11 hours prior to the study. Urine volume increased markedly during the infusion period (1.1 to 8.3 ml. per minute) and after infusion decreased. There was a prominent increase in C_{Osm} (1.71 to 5.03 ml. per minute) and also in C_{H_2O} (–0.65 to 3.71 ml. per

minute) and the osmotic U/P reached a minimum value of 0.55. Urine sodium and potassium excretion increased strikingly during the administration period (127 to 557 μEq per minute) and remained at relatively high levels after the infusion.

Infusion of hyperoncotic salt poor human albumin: (Table VIII.) Water diuresis was initiated by the ingestion of 1,500 ml. of water (from –110 minutes to –45 minutes) and was maintained by an oral intake of water in quantities slightly exceeding urinary excretion. Two hundred and fifty milliliters of 25 per cent salt poor human albumin was infused over a period of 45 minutes (100 ml. over 15 minutes followed by 150 ml. over 35 minutes).

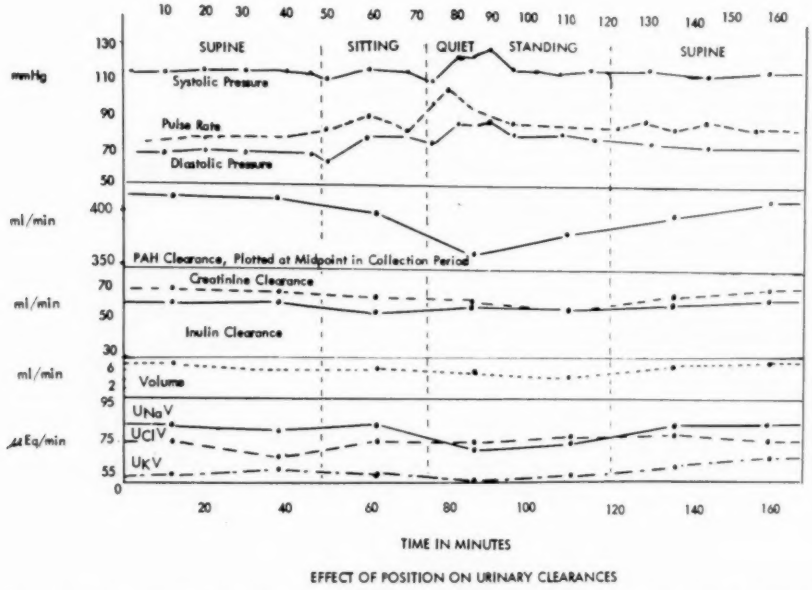

Fig. 1.—The effects of orthostatic position on C_{In}, C_{PAH}, C_{Cr}, water, and electrolyte excretion in the recipient.

C_{Cr} varied irregularly without apparent trend. Urine flow, C_{Osm}, and C_{H_2O} decreased during infusion and increased in subsequent periods. Osmotic urine to plasma ratios increased slightly over the last preinfusion control value and then decreased in follow-up samples. Sodium and chloride excretion decreased significantly during albumin infusion and increased during follow-up periods.

DISCUSSION

Effective renal plasma flow and glomerular filtration rate were found to be similar in each of the twins following the 1 stage bilateral nephrectomy on the recipient. These were within a normal range for a single kidney. The ability of the kidney to acidify and alkalinize urine, to concentrate and dilute urine, to respond to diurnal variation and to changes in body position were examined and found to be within normal ranges.

Kaplan and Rapoport[8] questioned the integrity of sodium excretion in the denervated kidney when they found the denervated organ frequently excreted more sodium than the intact organ. The majority of the denervated kidneys that excreted more sodium had slightly higher filtration rates than the contralateral intact kidneys. However, Bricker and coauthors,[9] in their studies of the integrity of sodium excretion in the autotransplanted kidney of the dog, found a decrease in filtration rate and in renal plasma flow in initial studies after transplantation but this defect did not persist. When sodium excretion was studied in these animals, excretion rates were found to be factored by their respective filtered loads, and the ratios were not consistently greater for transplanted kidneys. In several experiments they were less. Extracellular fluid volume expansion with 3 per cent sodium chloride resulted in excretion of sodium rich urine by both kidneys and expansion of extracellular fluid volume with isotonic solution revealed no abnormality of sodium excretion. Bricker and co-workers[2] described abnormalities in sodium excretion in the human homotransplanted kidney after acute extracellular fluid volume alterations. The patient in the present study differed from their patient. He had a shorter period of renal ischemia and a shorter period elapsed before nephrectomies of the diseased organs. Accordingly, we felt that further studies of the integrity of sodium excretion were indicated. To date Bricker's patient[2] is the first and only individual treated and studied in this manner.

Strauss and associates[10] have shown that the normal hydrated human subject in the supine position responds to rapid infusion of 2 L. of hypotonic sodium chloride by an increase in urine sodium excretion ($U_{Na}V$) despite the decreased plasma sodium ion concentration. Bricker and co-workers[2] found that their patient with a transplanted human kidney had a progressive decrease in total solute, sodium, and chloride excretion rates. C_{Cr} decreased moderately and urine flow showed only minor changes. In contrast to this, our recipient responded by increases in sodium excretion and free water clearance whereas osmolar clearance increased only slightly.

The normal human subject in the hydropenic state responds to acute intravenous infusion of isotonic saline by elaboration of an isotonic or hypertonic urine and by a modest increase in excretion of sodium and water; however, if a single dose of 2,000 ml. of water is given from 8 to 12 hours prior to the saline infusion, large amounts of both sodium and water are excreted and the urine becomes hypotonic.[11, 12] Bricker and co-workers[2] discovered that their transplant recipient in the hydropenic state responded by a significant increase in free water clearance from −1.2 ml. per minute to 4.1 ml. per minute, with a markedly hypotonic urine (osmotic U/P ratio of 0.36). The maximum $U_{Na}V$ was 135 μEq per minute. In the prehydrated state he responded normally. In contrast to this, our recipient, in the hydropenic state, responded to the intravenous infusion of 3,000 ml. of isotonic saline by a decrease in free water clearance, an increase in urine sodium excretion, an increase in osmolar clearance, and an elaboration of a hypertonic urine, which are normal responses. In the prehydrated state he responded normally to rapid infusion of isotonic saline by elaborating a hypotonic urine with an increase in osmolar clearance and in

free water clearance. The normal supine human subject responds to intravenous administration of 25 per cent salt poor albumin by a decrease in solute and water excretion[13] and by a more marked antidiuresis when the albumin is infused rapidly.[14] In our recipient there were decreases in urine flow, C_{H_2O} and C_{Osm} during albumin infusion. There were increases in C_{H_2O} and urine flow and a slight increase in C_{Osm} during follow-up periods. Sodium and chloride excretion decreased appreciably during albumin infusion and increased in the follow-up periods. Osmotic urine to plasma ratios increased slightly during the infusion and later decreased slightly.

The recipient has maintained normal body weight and plasma electrolyte concentrations over a period of many months. Acute changes in volume both with and without changes in tonicity produced changes in water excretion and electrolyte excretion rates similar to those which are found in normal subjects. These data suggest that renal nerves are not a part of the efferent limb of the volume control system.

SUMMARY AND CONCLUSIONS

A report of a successfully homotransplanted kidney in an identical twin was presented and the functional integrity of the denervated kidney after simultaneous bilateral nephrectomy was investigated under these experimental conditions. It is concluded:

1. That renal nerves are not necessary for the chronic regulation of extracellular fluid volume.

2. That the possibility exists that the abnormalities observed by others may have been due to the longer ischemic period for the transplanted kidney or to a longer lapse between transplantation of the kidney and nephrectomies of the diseased organs, or both.

3. That renal nerves are not actually necessary for the acute regulation of extracellular fluid volume.

4. The renal nerves do not appear to be essential for the normal response of the kidney in the other diversified functions tested in this investigation.

5. That the homologous transplanted kidney may maintain homeostasis.

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EFFECT OF PREDNISONE ON BLOOD COAGULATION TIME IN PATIENTS ON DICUMAROL THERAPY

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Previous experience with ACTH (adrenocorticotrophic hormone) and corticosteroids has shown that these substances when administered by injection or by mouth will shorten the blood coagulation time within a number of hours. It seemed of interest to determine the effect of prednisone on the clotting time when this had been prolonged by Dicumarol (bishydroxycoumarin) therapy. A significant decrease of the whole blood coagulation time was observed after oral administration of 10 mg. of prednisone in patients who had received Dicumarol for several days. The mean silicone coagulation time in these patients who had received Dicumarol before administration of prednisone was found to be 28.10 ± 2.59 minutes; 2 hours after the administration of prednisone it was 23.90 ± 2.23 minutes; 4 hours after administration it was 22.00 ± 1.85 minutes. The clotting accelerating mechanism of prednisone and its possible therapeutic implications are briefly discussed.

IT HAS been demonstrated in a previous study[1] dealing with the effect of corticotrophin, cortisone, and prednisone on coagulation time, that prednisone, when administered orally in therapeutic doses, will usually accelerate whole blood coagulation time. This effect will be particularly conspicuous when the determination is performed in siliconized tubes.[1] The influence of Dicumarol on blood coagulation has been investigated by many authors; it seems that not only prothrombin but also other coagulation factors are affected —mainly Factor VII (proconvertin) and another factor necessary for thromboplastin formation.[2-7] A prolongation of clotting time as determined in glass tubes has been observed after long-term treatment with anticoagulants,[2, 8] a phenomenon which we have also seen to take place—and to a much larger extent—in silicone coated tubes.

On the other hand, de Nicola[3] found an increase of Factor VII activity after administration of cortisone and corticotrophin; Chatterjea and Solomon[9] and Fazlullah[10] observed that patients on corticosteroid treatment who received anticoagulants at the same time because of thromboembolic complications would need larger doses of ethyl biscoumacetate. This requirement of larger doses of anticoagulants would decrease after cessation of corticosteroid therapy. Consequently it seemed of interest to investigate the influence of prednisone on the clotting time prolonged by Dicumarol.

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MATERIAL AND METHODS

The subjects of this investigation were 24 ward patients receiving Dicumarol because of thromboembolic diseases such as myocardial or pulmonary infarction or thrombophlebitis. They had not received prednisone before. The patients had been given Dicumarol long enough to depress prothrombin levels to 40 per cent or less of control values. They received a light breakfast before the experiment and care was taken to avoid hunger during the experiment by offering them tea and rusks. Prior to oral administration of 10 mg. prednisone an absolute blood eosinophil count was taken and the glass and silicone coagulation times of venous blood as well as prothrombin times were determined. Two and 4 hours after the administration of prednisone, glass and silicone coagulation times were again determined; a second eosinophil count was taken 4 hours after the administration of prednisone. In 12 of these patients, as a control study, the same examinations at the same time intervals were performed in which placebo tablets were used.

Prothrombin time was determined in several patients before, and 2 and 4 hours after administration of prednisone. Since in these preliminary experiments no significant change was observed, only the values obtained the morning before administration of prednisone are presented.

Clotting Time Determination.—About 12 ml. of venous blood was taken and used only when obtained by a technically satisfactory puncture. One milliliter of blood was put into each of 2 glass and 2 siliconized test tubes (size 10 by 100 mm.) in a water bath at 37° C. Syringes, needles, and tubes were siliconized* and used only once. The moment 50 per cent of the blood entered the syringe was taken as the zero point. The first glass tube was tilted every 30 seconds; the first silicone coated tube was tilted for the first time after 5 minutes, subsequently every 1 minute. The second glass and siliconized tubes were only tilted after the first ones had shown clotting and the time of clotting in these tubes was recorded and used for evaluation. The first few milliliters of blood which entered the syringe was discarded.

In the course of previous work in this field[1, 11] and through the literature we had become familiar with the intricacies of clotting time determinations, especially in siliconized tubes. This method has recently been strictly evaluated by Merskey and Nossel[12] who pointed out a number of difficulties of this method and of its evaluation when comparing results obtained from different patients under varying circumstances. We have carried out double determinations on samples taken through 1 venipuncture and the maximum difference observed between 2 tubes was occasionally 1 minute, 30 seconds. Our technician, thoroughly familiar with this type of determination, has mostly been unaware whether prednisone or placebo had been given to a patient under study. We have made it a point in this, as in our previous work,[1] to eliminate results from patients who seemed to be very anxious because of the repeated taking of blood samples.

Eosinophil Counts.—Eosinophil counts were done with 0.01 per cent eosin—5 per cent acetone stain in distilled water.

Prothrombin Time.—Prothrombin time was determined by the Quick method and using a dried rabbit brain thromboplastin (Difco) preparation and calcium chloride, 0.02 molar solution. Fresh human normal plasma diluted with 0.85 per cent sodium chloride was used to make up dilution curves for expressing prothrombin activity in per cent of normal concentration.

RESULTS

Table I presents the results of the examinations performed before and after administration of 10 mg. prednisone in 24 patients who had received Dicumarol.

*Silicone resin, Driflm 9986, General Electric Corp.

TABLE I. RESULTS OF GLASS (G.C.T.) AND SILICONE (S.C.T.) COAGULATION DETERMINATIONS, EOSINOPHIL COUNTS, AND PROTHROMBIN TIME BEFORE AND AFTER ORAL ADMINISTRATION OF 10 MG. PREDNISONE IN PATIENTS RECEIVING DICUMAROL

NO.	PATIENT	SEX	G.C.T. (MINUTES)			S.C.T. (MINUTES)			TOTAL EOSINOPHIL COUNT IN MM.^{3}		PROTHROMBIN CONCENTRATION (%)
			BEFORE	2 HOURS AFTER	4 HOURS AFTER	BEFORE	2 HOURS AFTER	4 HOURS AFTER	BEFORE	4 HOURS AFTER	
1	M. S.	F	6.5-10	8.5-11	14.5-17	42-62	21-57	16-28	209	196	40
2	G. D.	M	8.5-11	7-11.5	7-10	18-22	9-12	16-20	407	77	31
3	G. J.	M	9.5-13	8-13.5	6-9.5	17-29	16-24	12-17	143	88	30
4	G. R.	F	11-13	8-11	7.5-10	13-18	14-16	13-20	220	22	23
5	R. Ch.	F				21-26	15-20	7-10	176	66	46
6	S. L.	F	9.5-14	9-12.5	9-13	25-43	21-35	20-36	154	120	22
7	F. M.	M	11.5-16	9-13	9-12	27-60	28-48	25-43	143	132	24
8	S. N.	F	7-9.5	6-8.5	6.5-10	17-30	15-20	17-24	165	55	25
9	J. J.	M	5.5-8	5.5-8	5-6.5	13-19	12-14	8-11	220	11	42
10	T. S.	M				19-25	10-17	14-17	77	55	46
11	W. G.	M	8-12.5	7-10.5	7-10.5	13-24	19-25	18-32	110	44	25
12	L. K.	M	5-7	5-7	5.5-8	9-11	9-9	10-12	132	132	44
13	S. Z.	F	9.5-12	8-10	8-10.5	28-45	27-42	26-38	44	44	25
14	L. S.	M	9-11	9-10	6-8	24-35	22-38	24-30	99	99	30
15	H. B.	M	9-12.5	7.5-11	6.5-8	17-23	16-24	12-23	363	231	27
16	L. N.	M	6.5-8.5	7.5-10.5	7-10.5	16-19	13-17	12-14	187	110	20
17	G. P.	F	9-12	7-11	6.5-9	22-28	14-19	14-17	209	99	33
18	Z. H.	F	8-10	6.5-9	6-7.5	15-22	13-19	16-23	198	110	44
19	G. B.	M	5-8	5-7.5	5-6	16-22	12-17	13-18	110	110	48
20	K. N.	M	5.5-7.5	7-7.5	6.5-9.5	19-27	19-27	20-24	55	11	30
21	M. A.	M	9-13	7.5-11.5	10-14	14-18.5	14-21	18-29	154	253	26
22	D. Ch.	F	6-8.5	5.5-7.5	5-7.5	17-21	14-17	9-14	440	253	21
23	K. J.	M	6.5-9.5	6-8	5.5-8.5	22-27	16-19	14-18	352	220	30
24	P. J.	M	5.5-8	5.5-6.5	6-8	13-19	11-16	9-12	198	110	37

TABLE II. RESULTS OF GLASS (G.C.T.) AND SILICONE (S.C.T.) COAGULATION DETERMINATIONS, EOSINOPHIL COUNTS, AND PROTHROMBIN TIME BEFORE AND AFTER ORAL ADMINISTRATION OF PLACEBO IN PATIENTS RECEIVING DICUMAROL

NO.	PATIENT	SEX	G.C.T. (MINUTES)			S.C.T. (MINUTES)			TOTAL EOSINOPHIL COUNT IN MM^3		PROTHROMBIN TIME (%)
			BEFORE	2 HOURS AFTER	4 HOURS AFTER	BEFORE	2 HOURS AFTER	4 HOURS AFTER	BEFORE	4 HOURS AFTER	
1	F. M.	M	5.5-8.5	5.5-8.5	6-8.5	13-17		13-16	88	110	52
2	S. N.	F	6.5-8	6-8	6.5-8	14-16	11-11	14-18	132	143	76
3	J. J.	M	7-9	5.5-7	6.5-8.5	11-21		12-16	77	44	33
4	G. P.	F	7-11	7-10.5	7.5-10	23-40	24-43	23-42	187	209	22
5	Z. H.	F	6-8	6-9	6-8.5	18-23	21-32	17-25	209	231	30
6	G. B.	M	6-8	6-8	5-7.5	10-14	10-15	17-25	88	209	34
7	K. N.	M	9-10.5	6.5-8	7-9.5	37-42	21-32	21-31	187	110	28
8	M. E.	M	8.5-12	9-13.5	8.5-12	23-28	25-31	21-26	242	242	26
9	D. Ch.	F	5-7	5-7	5-7	13-16	12-15	12-15	495	462	37
10	K. J.	M	6-9.5	7-10	8-11	16-19	12-16	15-24	341	319	43
11	P. J.	M	6-8	6-8	5.5-7.5	15-20	12-16	12-15	231	275	81
12	Ch. J.	F	10.5-13	8-12	8.5-12	18-31	16-32	17-27	176	297	25

The results of the control studies—before and after placebo administration—are given in Table II. The average coagulation times observed and the average differences before and 2 and 4 hours after administration of placebo and prednisone, respectively, are summarized in Table III. The mean silicone time has been previously found to be 16.22 ± 0.542 (standard deviation of the mean) in a control group who had not received Dicumarol.

TABLE III. SUMMARY OF AVERAGE COAGULATION TIMES AND DIFFERENCES IN PATIENTS RECEIVING DICUMAROL BEFORE AND 2 AND 4 HOURS AFTER ORAL ADMINISTRATION OF PLACEBO AND 10 MG. PREDNISONE

COAGULATION TIME (HOURS)	NO. OF CASES STUDIED	NO. OF CASES WITH DECREASES OF COAGULATION TIME	AVERAGE COAGULATION TIME: BEFORE ADMINISTRATION ± S.D. OF THE MEAN	AVERAGE COAGULATION TIME: AFTER ADMINISTRATION ± S.D. OF THE MEAN	AVERAGE DIFFERENCES ± S.D. OF THE MEAN
Controls (Placebo).—					
G.C.T. 2	12	4	9.37 ± 0.54	9.13 ± 0.55	0.16 ± 0.28
G.C.T. 4	12	5	9.37 ± 0.54	9.16 ± 0.50	0.20 ± 0.20
S.C.T. 2	10	5	23.50 ± 2.85	24.30 ± 3.42	0.50 ± 1.62
S.C.T. 4	12	7	23.50 ± 2.85	23.33 ± 2.32	0.58 ± 1.62
Prednisone (10 mg.)					
G.C.T. 2	22	15	10.65 ± 1.07	9.84 ± 0.40	0.96 ± 0.26
G.C.T. 4	22	15	10.65 ± 1.07	9.70 ± 0.54	0.95 ± 0.53
S.C.T. 2	24	20	28.10 ± 2.59	23.90 ± 2.23	4.27 ± 0.76
S.C.T. 4	24	18	28.10 ± 2.59	22.00 ± 1.85	6.10 ± 1.80

S.D. = standard deviation; G.C.T. = glass coagulation time; S.C.T. = silicone coagulation time.

TABLE IV. STATISTICAL EVALUATION OF EFFECTS OF 10 MG. PREDNISONE ON GLASS (G.C.T.) AND SILICONE COAGULATION TIME (S.C.T.) IN PATIENTS RECEIVING DICUMAROL

	G.C.T.		S.C.T.	
	2 HOURS	4 HOURS	2 HOURS	4 HOURS
T	34	42	13	33
n	19	20	23	23
p	$0.005 < p < 0.01$	$0.005 < p < 0.01$	$p < 0.005$	$p < 0.005$

T = matched samples; n = number of cases with before-after difference different from zero; p = probability factor.

The difference between readings before and 2 and 4 hours after prednisone administration both in glass and silicone coated tubes has been tested by Willcoxon's matched samples (T) test. The results of this test are given in Table IV. The one tailed test procedure was used since the hypothesis tested is that prednisone lessens coagulation time. It is easily seen from these tables that the differences are significantly different from zero. In the control group differences between the readings before and after placebo administration were very small and randomly distributed to the positive and negative sides.

DISCUSSION

Acceleration of whole blood coagulation time by prednisone in most patients who had received Dicumarol is evident when the examinations are performed in silicone coated tubes. Prednisone in the dose applied seems

consequently to obviate the effect of anticoagulant treatment. This observation is in line with previous reports which state that the corticosteroids can produce a state of hypercoagulability which may then predispose to thromboembolic complications.[13, 14] It is difficult, however, to relate the thromboembolic episodes occurring in patients receiving hormonal treatment to the administration of these substances alone, because such patients may easily become prone to develop these complications through the combined effect of bed rest and many other factors connected with the underlying disease.

It is impossible to draw any conclusions as to which of the clotting factors is or are influenced by administration of prednisone from this study. Since proconvertin deficiency apparently determines the prolongation of clotting time in patients treated with Dicumarol, it may be assumed that prednisone mobilizes or replaces convertin activity, an effect similar to de Nicola's observation for ACTH and cortisone.[3]

Incidentally, the prolongation of the silicone coagulation time by Dicumarol which usually appears a few days after the beginning of anticoagulant treatment may serve in the evaluation of this regime. Mayer and Connell have demonstrated that Dicumarol,[15] ethyl biscoumacetate,[16] and Sintrom[17] prolong coagulation time as examined in glass tubes by a standardized method.[18] Our results point to the same direction.

SUMMARY

Whole blood coagulation time was determined in glass and silicone coated test tubes in 24 patients who had received Dicumarol before and 2 and 4 hours after administration of 10 mg. of prednisone; 12 of these individuals served as controls.

Prolongation of the silicone coagulation time appeared regularly in the course of Dicumarol treatment. The mean silicone coagulation time in the 24 patients who had received Dicumarol before the administration of prednisone was 28.10 ± 2.59 minutes (standard deviation of the mean), whereas the mean silicone coagulation time as determined by us previously[1] in control patients without anticoagulation treatment was 16.22 ± 0.542 minutes.

The administration of 10 mg. of prednisone produced a statistically significant acceleration of the silicone coagulation time prolonged by Dicumarol. The mean silicone coagulation time 2 hours after administration of prednisone was found to be 23.9 ± 2.23 and 4 hours after prednisone was given, it was 22.0 ± 1.85 (standard deviation of the mean).

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DIHYDROVITAMIN K_1 BIPHOSPHATE IN BISHYDROXYCOUMARIN INDUCED HYPOPROTHROMBINEMIA

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Dihydrovitamin K_1 biphosphate, a water soluable salt, has been reported to be an effective antagonist to drug induced hypoprothrombinemia, comparable to, if not superior to oil soluble vitamin K_1. This water soluble salt was administered to hospitalized patients rendered hypoprothrombinemic with bishydroxycoumarin in doses varying from 5 to 50 mg. orally, 10 to 50 mg. intravenously, and 50 mg. intramuscularly and 50 mg. subcutaneously. Prothrombin times observed over 24 hours demonstrated this vitamin to be quite ineffective when comparisons to a control group were made. A definite positive effect greater than the control could be found only after 50 mg. intravenously or subcutaneously was given; all other dose ranges and routes were lacking in prothrombin time response.

THE ever-increasing clinical use of hypoprothrombinemia inducing anticoagulants has emphasized the need for a readily available antidote. Such an antidote is required under numerous circumstances (e.g., spontaneous hemorrhagic complications caused by an overdose of anticoagulant drug or drug hypersensitivity, hemorrhage caused by accidental injury while under such therapy, or the unexpected appearance of a new medical or surgical illness superimposed upon the patient's initial disease and urgently requiring the discontinuance of the induced hypoprothrombinemia). Phytonadione (or oil soluble vitamin K_1) is widely accepted as the most effective antidote for drug induced hypoprothrombinemia.[1, 2] It is administered orally in doses ranging from 10 to 50 mg.,[3, 4] or parenterally as an oil in water emulsion, usually administered intravenously in 50 mg. doses.[3, 4] The use of an oily material has some minor disadvantages, however. There are mechanical difficulties inherent in the use of the injectable emulsion (e.g., instability of its colloidal state with "breaking" of the emulsion and loss of the colloidal dispersion). Another handicap to its simplicity of use is that the emulsion requires dilution before injection. The pharmaceutically ideal preparation should be water soluble and be capable of being injected intravenously without dilution.

The phytonadione tablet for oral administration has been a useful preparation, but there is a theoretic objection in that as an oil soluble material it would

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not be well absorbed in the clinical situation of complete biliary obstruction. A water soluble material theoretically should not require the emulsifying action of bile salts for intestinal absorption.

Mushett and associates[5] have reported upon their experience with a water soluble disodium salt of vitamin K_1, the formula of which is 2-methyl-3-phytyl-1, 4-naphthohydroquinone-1, 4-disphosphate (dihydrovitamin K_1 biphosphate). They administered this material orally, intramuscularly, and intravenously into dogs rendered hypoprothrombinemic by bishydroxycoumarin (Dicumarol) or diphenadione (Dipaxin) in short term experiments. They reported that equimolar intravenous and oral doses of the dihydrovitamin K_1 biphosphate and of vitamin K_1 emulsion exhibited like activity in reversing the drug induced hypoprothrominemia, whereas at equimolar dosage levels, given intramuscularly, they felt the water soluble compound was superior. Bole and Duff[6] tested this compound in three healthy volunteers rendered hypoprothrombinemic with bishydroxycoumarin and found a significant effect upon the prothrombin time within 1 hour, and upon the Factor VII levels within 1½ hours. These workers also used it in the clinical situations of drug induced excessive hypoprothrombinemia, one a 78-year-old woman with profuse gastrointestinal hemorrhage and the other a woman with a prothrombin time of 59 seconds. Excellent responses were obtained in both with doses of 40 and 60 mg., respectively, given intravenously, obtaining safe prothrombin levels in 5 and 3 hours, respectively.

With these enthusiastic reports in mind, we felt a clinical screening of the efficacy of this drug in various dose ranges and in the usual modes of administration was indicated.

METHOD

The studies were performed successfully upon 41 adults who previously had been under anticoagulant therapy, using bishydroxycoumarin for relevant illnesses often associated with thromboembolic phenomena. Twenty-eight of this group were hospitalized for acute myocardial infarctions, 11 for acute coronary insufficiency, and 2 for acute pulmonary infarction. Blood prothrombin times were determined daily. Bishydroxycoumarin was discontinued the day before testing, usually after weeks of stabilized levels of hypoprothrombinemia, with levels maintained at 50 per cent (22 to 23 seconds) or less. On the morning of the day of testing, determination of the prothrombin times was done just prior to the administration of dihydrovitamin K_1 biphosphate, then 2, 4, 6, 8, and 24 hours subsequent to this time. Seven additional patients were used as controls. All procedures were done similarly, with the exception of the administration of the vitamin K_1. Oxalated blood was used throughout, and the prothrombin times were determined by the Link-Shapiro modification of the Quick method, using Solu-Plastin (solution of thromboplastin derived from fresh brain and lung tissue).

RESULTS

The results of the experiment are summarized in Tables I and II. The individual control prothrombin times in seconds were used as the base line value, and deviations from this value were averaged as mean positive or negative values. For example, a fall in prothrombin time of 5 seconds is recorded as

–5 and a rise of 8 seconds as +8. Since the bishydroxycoumarin was discontinued for 24 hours prior to testing, a moderate fall in the prothrombin times over the succeeding 24 hours of the test, or 48 hours after the last dose of anticoagulant is to be expected in this series of 7 subjects. The average fall in 24 hours was 10 seconds. An accelerated fall toward normal levels should be obtained with a potent vitamin K_1.

Table I charts the results after oral administration of 5, 10, and 50 mg. of the water soluble vitamin. There is no discernible difference between these dose ranges and the control series over the 24 hours of observation. Falls in prothrombin times of 5 to 10 seconds were observed after 24 hours and these were distributed in a random fashion despite the dose variations.

TABLE I. RESPONSE OF BISHYDROXYCOUMARIN (DICUMAROL) INDUCED HYPOPROTHROMBINEMIA TO ORALLY ADMINISTERED DIHYDROVITAMIN K_1 BIPHOSPHATE

TIME (HR.)	CONTROL (7)* MEAN FALL (SEC.)	5 MG. (5) MEAN FALL (SEC.)	10 MG. (5) MEAN FALL (SEC.)	25 MG. (5) MEAN FALL (SEC.)	50 MG. (4) MEAN FALL (SEC.)
2	–2	+1	0	+1	–8
4	–3	–2	–4	+3	+6
6	–5	–2	0	+2	+6
8	+5	–2	–1	+4	+2
24	–10	–8	–10	–5	–5

*Numbers in parentheses are numbers of patients tested.

TABLE II. RESPONSE OF BISHYDROXYCOUMARIN (DICUMAROL) INDUCED HYPOPROTHROMBINEMIA TO PARENTERALLY ADMINISTERED DIHYDROVITAMIN K_1 BIPHOSPHATE

TIME (HR.)	CONTROL (7)* MEAN FALL (SEC.)	10 MG. I.V. (2) MEAN FALL (SEC.)	25 MG. I.V. (5) MEAN FALL (SEC.)	50 MG. I.V. (5) MEAN FALL (SEC.)	50 MG. I.M. (5) MEAN FALL (SEC.)	50 MG. S.C. (5) MEAN FALL (SEC.)
2	–2	0	0	–1	+1	0
4	–3	+4	–2	–3	+3	–3
6	–5	+2	–3	–7	+1	–3
8	–5	–2	–5	–8	–5	–4
24	–10	–10	–9	–15	–9	–21

I.V. = intravenous. I.M. = intramuscular. S.C. = subcutaneous.
*Numbers in parentheses are numbers of patients tested.

Table II charts the results after the parenteral administration of 10, 25, and 50 mg. intravenously, 50 mg. intramuscularly, and 50 mg. subcutaneously. This table demonstrates a definite response as compared to the control only after 24 hours subsequent to the intravenous and subcutaneous administration of 50 mg. doses. The smaller intravenous doses were ineffective, as was the intramuscular dose of 50 mg. These minimal changes are comparable to the minimal declines after the oral doses and do not differ from the control series. No significant untoward reaction was experienced by any patient. Two subjects had a concurrent elevation of temperature to 101° F. in the day of parenteral administration of the vitamin without other symptoms. The vitamin was used therapeutically in only one instance, when 50 mg. was given subcutaneously to a subject with gross hematuria and hemoptysis without effect over a 24 hour

observation period. At this time, 50 mg. of vitamin K_1 emulsion was given intravenously with a rapid amelioration of the bleeding diathesis.

DISCUSSION

It is not unusual for the effect of drugs to vary according to the species used for testing, in that the same drug may be quite effective in one species of laboratory animal, yet relatively impotent in another animal or in the human. Although the initial reports on the use of dihydrovitamin K_1 biphosphate as antidote to bishydroxycoumarin were favorable, the major experiments were limited to the dog.[5] The clinical screening of this drug to counter the hypoprothrombinemia induced by bishydroxycoumarin in the human failed to confirm its therapeutic effectiveness with dose ranges comparable to those recommended for phytonadione by oral and parenteral routes. The mechanical faults of the emulsion in clinical situations where it is required are minor and are more than overcome by its demonstrated superiority over the water soluble forms of vitamin K in such circumstances. Syncopal episodes are experienced on occasion, but major catastrophic reactions are exceedingly rare. For the present, it does not seem warranted to use the dihydrovitamin K_1 biphosphate clinically, except for specific investigative purposes.

SUMMARY

1. Dihydrovitamin K_1 biphosphate, a new water soluble salt of vitamin K_1, was screened clinically for effectiveness as an antidote to bishydroxycoumarin in patients rendered hypoprothrombinemic with this drug.

2. This vitamin is ineffective taken orally in doses ranging from 5 to 50 mg. over a 24 hour observation period.

3. In similar observations this vitamin demonstrates some effectiveness parenterally in doses of 50 mg. given intravenously or subcutaneously. This same dose given intramuscularly was ineffective as were intravenous doses smaller than 50 mg.

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BLOOD COAGULATION DURING SLEEP

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WITH THE TECHNICAL ASSISTANCE OF DOROTHY HEARNE

No differences between the waking and the sleeping state could be demonstrated in the following parameters of blood clotting: Quick prothrombin, prothrombin-proconvertin (Owren), proaccelerin, Russell's viper venom time, partial thromboplastin time, fibrinogen, platelet count, euglobulin lysis time, and thromboplastin generation test.

CHANGES in blood clotting during sleep have been reported in patients with paroxysmal nocturnal hemoglobinuria,[1] in animals during hibernation,[2, 3] and in normal subjects.[4] In the first situation such changes are said to contribute to the nocturnal hemolysis. These changes are reported to consist of an increase in the accelerator globulin (proaccelerin) activity, and in Factor VII (proconvertin) activity.[1] Such changes are similarly held to occur in the normal person, but we have been unable to find the data upon which this conclusion is based.[4] In hibernating animals there is a prolongation of the clotting time[2] and prothrombin time.[3] Because the data in humans were unconvincing and at variance with what one might expect by analogy, we felt that a systematic investigation of blood clotting in the sleeping human would give useful information.

METHODS

The subjects were normal individuals or patients without known involvement of the hematopoietic system. Each subject reported to the laboratory late in the evening without special preparation. A comfortable bed in a dark environment was provided. No sedative drugs were used. A polyethylene catheter was placed in an accessible arm vein and the first "waking" sample was taken. The subject was then allowed to sleep. Samples were taken at hourly intervals with every effort made to avoid disturbing the patient. Between samples the catheter was filled with heparin in saline (1 mg. per milliliter) to keep it patent. Over the course of the entire night not more than 10 mg. was injected and, in general, approximately 5 mg. was used. Prior to obtaining the sample, the catheter was flushed by the removal of 10 to 50 ml. of blood. The sample was obtained in a silicone coated syringe and transferred to tubes which contained the appropriate anticoagulant. For measurement of fibrinolysin, the sample was taken in a syringe which contained

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3.8 per cent sodium citrate as described.[5] Depending upon responsiveness, the subject was graded as awake, intermediate, or sound asleep, and the sample taken was so marked. A "waking" sample was always taken at the end of the night. In three subjects, because of blockage of the catheter during the night, the morning sample was taken by direct venipuncture. Samples were discarded if blood flow was not free.

Clotting tests were performed by the following methods: one stage prothrombin (Quick)[6] modified by the use of Owren's thromboplastin,[7] prothrombin-proconvertin (Owren),[7] specific proaccelerin assay,[8] prothrombin (Russell's viper venom method),[7] also considered to be sensitive to changes in the Stuart-Prower factor,[9] partial thromboplastin time (using crude brain extract as cephalin),[10] fibrinogen[6] modified by collecting the fibrin on filter paper, platelet count,[6] and euglobulin lysis time.[5]

Thromboplastin generation tests were performed using 4 basic reagents in the generating mixture: adsorbed plasma, serum, crude brain extract (cephalin) as a platelet substitute, and $CaCl_2$. After these are mixed the generation of thromboplastin is followed by adding aliquots to a test plasma at intervals. The clotting time of the test plasma reflects both the thromboplastin-like and thrombin-like activity generated in the test system. Since there is very little prothrombin in the generating system, the test primarily follows thromboplastin generation. In order to test various factors involved in thromboplastin generation the substrate plasma, the cephalin, and the calcium were kept constant and the other reagents varied as follows (each test being run on repeated samples throughout the night):

1. *For Evaluation of Plasma Factors (Antihemophilic Globulin, Proaccelerin).*—Oxalated plasma from the subject was adsorbed with $BaSO_4$ (100 mg. per milliliter) and was used as the only variable in the generation system. The other components of the system were: (a) eluate of $BaSO_4$ which had previously been mixed with normal serum (this eluate is similar to that described by Hjort[11] and serves as a stable source of plasma thromboplastin component and Stuart-Prower factor. It also contains small amounts of prothrombin), (b) cephalin,[10] and (c) $CaCl_2$.

2. *For Evaluation of Serum Factors (Plasma Thromboplastin Component, Stuart-Prower Factor).*—A single normal plasma was adsorbed with $BaSO_4$ and frozen. It was subsequently used in all tests as a source of antihemophilic globulin and proaccelerin. The serum reagent originated from the subject as follows: Blood was added from the syringe to each of three glass tubes (3 ml. each). The tubes were inverted repeatedly until the blood clotted. They were incubated at 37° C. for 1 hour after which the serum was separated by centrifugation and incubated an additional 5 hours. The 3 sera were then pooled and frozen until tested. Cephalin and $CaCl_2$ completed the system.

3. *Over-all Thromboplastin Generation (Dependent on all of the Foregoing Plus Plasma Thromboplastin Antecedent).*—Over-all thromboplastin generation was estimated by using the subject's oxalated adsorbed plasma prepared as in *1.* and serum prepared as follows: Three milliliters of blood was added from the syringe to each of 2 tubes which contained 0.1 ml. thromboplastin. The tubes were forcibly inverted 8 times, incubated for 30 minutes at 37° C., and the serum was separated by centrifugation. The sera were actively stirred with small pledgets of glass wool at the beginning and at the end of an additional 30 minute incubation. They were then placed on ice and the test was run within 30 minutes. A separate curve was determined for each serum tube and the average was taken.

Generation tests were performed with the following dilutions:

For plasma factors: (a) adsorbed plasma 1:20, and (b) serum eluate 1:10. (This corresponds to approximately the same activity as the parent serum.)

For serum factors: (a) adsorbed plasma 1:10, and (b) serum 1:20.

For over-all generation: (a) adsorbed plasma 1:10, and (b) serum 1:20.

Cephalin 1:200 and $CaCl_2$ 0.025M was used in all tests. Other conditions of the test system have been described.[12]

For convenience of reporting each generation curve is represented by its minimum clotting time (the fastest clotting time representing the peak activity), and by the incubation time (the time from the start of the reaction until peak activity is reached).

RESULTS

As is indicated in Tables I and II, there was no significant change in the parameters of clotting which were studied. The average values are given. It may be stated further that when individual records are followed throughout the night, there was no consistent trend.

TABLE I. CHANGES IN CLOTTING TESTS DURING SLEEP

		AWAKE			INTERMEDIATE			ASLEEP		
TEST	PATIENT	NO. OBS.	AVERAGE	TOTAL AVERAGE	NO. OBS.	AVERAGE	TOTAL AVERAGE	NO. OBS.	AVERAGE	TOTAL AVERAGE
One stage prothrombin (Quick) (sec.)	V.	2	12.1		1	11.8		2	11.7	
	A.	2	14.1		2	14.0		2	14.1	
	C.	2	14.0		1	13.3		1	14.1	
	E.	2	14.7		—	—		6	15.2	
				13.7			13.0			13.8
Prothrombin-proconvertin (Owren) (sec.)	V.	2	25.7		1	25.9		2	25.7	
	C.	2	32.9		1	31.5		1	30.2	
	E.	2	33.8		—	—		5	33.8	
				30.8			28.7			29.9
Proaccelerin (sec.)	V.	2	23.2		1	23.6		2	22.6	
	A.	2	27.0		2	26.3		2	25.5	
	C.	2	26.4		1	25.7		1	27.5	
	E.	2	27.0		—	—		5	27.9	
				25.9			25.2			25.9
Prothrombin-Russell's viper venom (sec.)	A.	2	27.6		1	26.4		3	26.5	
	C.	2	23.6		1	23.5		1	22.3	
	E.	2	33.6		—	—		4	33.7	
				28.3			24.9			27.5
Partial thromboplastin time (sec.)	V.	2	57.5		1	65		2	60	
	A.	3	68		1	75		2	67.5	
	C.	2	60		1	60		1	60	
	E.	2	87.5		—	—		5	76	
				68			67			66
Platelet count (thousands/mm.^{3})	P.	2	196		1	174		2	200	
	Ph.	4	261		—	—		2	251	
				228						226
Euglobulin lysis time (min.)	P.	2	85		1	70		2	107	
	Ph.	4	207		—	—		2	301	
	M.	2	97		1	70		2	132	
	R.	2	217		—	—		2	225	
				152						191
Fibrinogen (mg./100 ml.)	V.	2	399		—	—		2	390	
	A.	2	208		2	202		2	178	
	C.	2	176		1	145		1	198	
	E.	2	390		—	—		5	336	
				293			174			276

DISCUSSION

The chief problem attendant upon a study involving the use of an indwelling venous catheter is that it is necessary to use an anticoagulant to keep the catheter patent throughout the study. As a control for this factor the experiment described includes values obtained during the waking state at the beginning, the middle, and the end of the night. Variation of individual tests might be

ascribed to insufficient flushing of the catheter. This is particularly true of the thromboplastin generation tests in which the serum reagent originated from the patient. In spite of this the results are reproducible enough to indicate that there is no essential difference in blood clotting between the normal person in the waking state and the normal person in the sleeping state. These results do not agree with those reported by Lasch and co-workers[1] and alluded to by Crosby.[4] In the former report the patient studied had paroxysmal nocturnal hemoglobinuria and the acceleration of coagulation noted may have been the result rather than the cause of the hemolysis.[13] In the latter report data are not available for comparison.

TABLE II. CHANGES IN THROMBOPLASTIN GENERATION TESTS DURING SLEEP

		AWAKE			INTERMEDIATE			ASLEEP		
TEST	PATIENT	NO. OBS.	AVERAGE	TOTAL AVERAGE	NO. OBS.	AVERAGE	TOTAL AVERAGE	NO. OBS.	AVERAGE	TOTAL AVERAGE
For plasma factors (minimum clotting time; sec.)*	M.	2	10.5		2	11.0		2	11.0	
	W.	3	14.0		2	14.0		2	13.0	
	B.	2	10.5		1	11.0		3	10.0	
				11.7			12.0			11.3
For plasma factors (incubation time; min.)*	M.	2	4.5		2	4.0		2	6.0	
	W.	3	6.0		2	7.0		2	6.5	
	B.	2	6.0		1	5.5		3	5.5	
				5.5			5.5			6.0
For serum factors (minimum clotting time; sec.)	M.	3	9.8		3	9.8		2	9.8	
	B.	2	13.0		1	12.5		3	10.2	
	W.	3	14.7		2	14.9		2	13.9	
				12.5			12.4			11.3
For serum factors (incubation time; min.)	M.	3	5.0		3	5.9		2	8.7	
	B.	2	5.5		1	6.0		3	5.0	
	W.	3	6.2		2	6.5		2	6.5	
				5.6			6.1			6.7
Over-all generation (minimum clotting time; sec.)	R.	2	11.0		1	11.5		1	13.0	
	M.	2	17.0		2	18.5		1	18.0	
				14.0			15.0			15.5
Over-all generation (incubation time; min.)	R.	2	8.0		1	6.0		1	4.0	
	M.	2	7.5		2	9.5		1	13.0	
				7.8			7.8			8.5

*See text for definition of minimum clotting time and incubation time.

The data on fibrinolytic activity are of interest. Buckell and Elliott[14] have demonstrated a diurnal variation in fibrinolysis. Others have shown an inhibition of fibrinolysis in association with an increase in lipemia.[15] The absence of shifts in cholesterol and phospholipids during sleep[16] in association with the absence of changes in lysis time are compatible with these data. The method used in this study was similar to that with which diurnal fluctuation was demonstrated.

The absence of coagulation changes in man during sleep may have no direct relationship to the changes during hibernation in animals. The findings of this study reinforce the widely held view that hibernation and physiologic sleep are basically distinct and different entities. As with all studies of hemostasis, the

methods used here are crude when compared with the processes believed to be occurring in vivo. The present evidence indicates however that there are no significant changes in blood coagulability associated with natural sleep.

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TREATMENT OF EXPERIMENTAL ATHEROSCLEROSIS IN THE RABBIT WITH L.D. ALPHA (DIMYRISTOYL) LECITHIN

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The effect of an intravenously infused synthetic L.D. alpha (dimyristoyl) lecithin was studied on plasma cholesterol concentrations and atheromatous infiltrations in five rabbits in whom atherosclerosis had been experimentally induced.

Infusion of 100 ml. of 0.8 per cent L.D. alpha lecithin in 5 per cent glucose and saline into atherosclerotic rabbits resulted in a transient rise of plasma cholesterol and phospholipid phosphorus and a gradual fall of hematocrit.

Histologic examination of the aortas of the rabbits in which experimental atherosclerosis had been produced and who had received five infusions of lecithin, showed marked resolution of atheromatous plaques as compared with noninfused atherosclerotic control rabbits.

THE PRESENCE of widely distributed phospholipids in tissues, and especially in the cells, has led to many theories as to the possible function of phosphatides. Recently, Friedman and co-workers[1, 2] noted that soy bean phosphatide mixture infused intravenously into rabbits caused hypercholesterolemia. Furthermore, it was observed that infusion of soy bean phosphatide into rabbits which had been previously made atherosclerotic produced resolution of the atheromatous infiltration of the rabbit aortas.[3]

In this article an attempt is made to study the effect of pure synthetic L.D. alpha (dimyristoyl) lecithin on atheromatous infiltration of rabbit aortas. This lecithin was chosen in this investigation for its outstanding property to form an aqueous emulsion, and because the lecithins are typical representatives of the plasma phospholipids. The information gained may lead to a better understanding of the role of one important member of the phosphatide family in relation to cholesterol metabolism and atherosclerosis.

MATERIALS AND METHODS

Rabbits.—Fifteen male rabbits, 5 to 7 weeks old and weighing 3½ to 4½ pounds were used.

Induction of Atherosclerosis.—Ten of these rabbits were placed on a diet of Purina chow* which contained 3 per cent cholesterol and 4 per cent cottonseed oil for a period of 11

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*The special high cholesterol rabbit chow used in the experiments was prepared by the Ralston Purina Company, St. Louis, Mo.

weeks. The chow and water were offered ad libidum. The weight and the plasma cholesterol of the rabbits were determined at the beginning of the experiment and twice during the course of the feeding (Table I). At the end of the high cholesterol feeding period the rabbits were returned to regular Purina chow for 30 days. Regular Purina rabbit chow was fed to 5 control rabbits at the same time.

L,D. Alpha (Dimyristoyl) Lecithin Emulsion.—Synthetic lecithin of known structure and configuration was prepared by modifying the Baer and Kates,[4] Baer and Maurukas[5] procedure. The yield so obtained ranged from 50 to 70 per cent of the theoretical (based on dimyristin glycerol).

Synthetic lecithin was found to be stable in the freezer compartment of a refrigerator for 12 months (at −15° C.). At the end of this period it started to show some hemolytic reaction on rabbit and human red blood cells. The impurity causing the hemolysis was removed by diazomethylysis[6] followed by recrystallization from ether.[7]

A 0.8 per cent emulsion was prepared fresh each morning by adding 0.8 Gm. of lecithin to 100 ml. of sterile 5.0 per cent glucose saline and shaking for 10 to 20 minutes at room temperature. The pH of the emulsion varied from 6.7 to 6.9. Filtration through a Berkefeld candle filter produced a bacteria-free emulsion. Bacteriologic investigation, however, showed the emulsion to be free from bacteria before filtration, and filtration was not used routinely.

An attempt was made to prepare lecithin emulsions of higher concentration; however, the viscosity of the emulsion was too high to be of use in intravenous infusion experiments.

Analytic Methods.—Total plasma cholesterol was determined by the method of Bloor and collaborators.[8] Total lipid phosphorus analysis was done according to the method of King,[9] and total fat determination according to the modified procedure of Zukerman and co-workers.[10]

TABLE I. WEIGHT AND BLOOD CHOLESTEROL LEVELS IN RABBITS DURING INDUCTION OF EXPERIMENTAL ATHEROSCLEROSIS BY HIGH CHOLESTEROL FEEDINGS

	INITIAL (PREFEEDING)		END OF FIRST MONTH		END OF SECOND MONTH		PREINFUSION (POSTFEEDING)	
RABBIT	WEIGHT	MG. % CHOLESTEROL	WEIGHT	MG. % CHOLESTEROL	WEIGHT	MG. % CHOLESTEROL	WEIGHT	MG. % CHOLESTEROL
1	3 lb.		4 lb.		5 lb.			
	11 oz.	35	14 oz.	172	8 oz.	302	—	—
2	3 lb.		5 lb.		6 lb.			
	10 oz.	34	6 oz.	491	6 oz.	1,030	—	—
3	3 lb.		5 lb.		6 lb.			
	11 oz.	92	3 oz.	388	10 oz.	1,120	—	—
4	3 lb.		4 lb.		6 lb.			
	12 oz.	63	13 oz.	118	1 oz.	2,640	—	—
5	3 lb.		4 lb.		4 lb.			
	15 oz.	33	10 oz.	1,080	11 oz.	1,750	—	—
6	4 lb.		5 lb.		5 lb.		6 lb.	
	1 oz.	24		77	8 oz.	717	5 oz.	17.6
7	3 lb.		5 lb.		6 lb.		7 lb.	
	12 oz.	109	8 oz.	190	12 oz.	2,240	8 oz.	100.0
8	4 lb.		5 lb.		4 lb.		5 lb.	
	3 oz.	54	8 oz.	535	15 oz.	846	4 oz.	39.6
9	4 lb.		5 lb.		6 lb.		7 lb.	
	2 oz.	23	1 oz.	174	14 oz.	537	9 oz.	43.0
10	4 lb.		4 lb.		5 lb.		6 lb.	
	2 oz.	54	1 oz.	140	11 oz.	575	6 oz.	31.0

The aorta, after being stripped of adventitia, was dried at an oven temperature of 100 to 105° C. until a constant weight was obtained. After grinding the dried tissue with alcohol-ether treated sand, it was extracted 3 times with 30 ml. (total 90 ml.) of hot alcohol-ether mixture (3:1). The insoluble residue was removed by centrifugation. The combined extracts

TABLE II. EFFECT OF L,D. ALPHA (DIMYRISTOYL) LECITHIN ON BLOOD CHOLESTEROL, PHOSPHOLIPID-PHOSPHORUS, AND HEMATOCRIT OF ARTERIOSCLEROTIC RABBITS

RABBIT	PREINFUSION HCT. (%)	LOWEST POST-INFUSION HCT. (%)	PREINFUSION CHOLESTEROL (MG. %)	AVERAGE OF POSTINFUSION CHOLESTEROLS (MG. %)	PREINFUSION PHOSPHO-LIPID (MG. %)	AVERAGE OF POSTINFUSION PHOSPHO-LIPIDS (MG. %)
6	40	17	17.6	69.0	2.2	6.9
7	30	25	100.0	115.0	5.6	8.7
8	33	22	39.6	69.0	3.5	6.0
9	38	19	43.0	67.0	1.1	6.6
10	37	16	31.0	60.0	4.1	5.5

were transferred to a 100 ml. volumetric flask and made up to 100 ml. with alcohol-ether mixture (3:1). Aliquots were analyzed for total lipid, cholesterol, and lipid phosphorus.

Procedure.—The external jugular veins of five atherosclerotic rabbits were cannulated according to the method described by Friedman and associates.[3] Morphine sulfate was used for anesthesia. Two days after cannulation, each rabbit was infused via the cannula over approximately a 4 hour period with 100 ml. of 0.8 per cent L,D. alpha (dimyristoyl) lecithin emulsion. The infusion was repeated at intervals of 4 to 6 days. Two rabbits of this group received 5 infusions. One rabbit received 4 infusions and two others received 3 infusions each. Blood samples were obtained before and at variable intervals after infusion. These were analyzed for total cholesterol, phospholipid phosphorus, and hematocrit (Table II).

At the end of the series of infusions the rabbits were permitted to rest for approximately 6 to 12 days. The 5 atherosclerotic rabbits which had been infused with lecithin, and the 5 atherosclerotic rabbits which served as controls were killed and the aortas were removed. Five normal rabbits were also killed and the aortas were used as controls. The degree of atherosclerosis was estimated grossly and microscopically.

After gross and microscopic examination, the remaining portions of the aortas were dried and analyzed for cholesterol, phospholipid phosphorus, and total fat content.

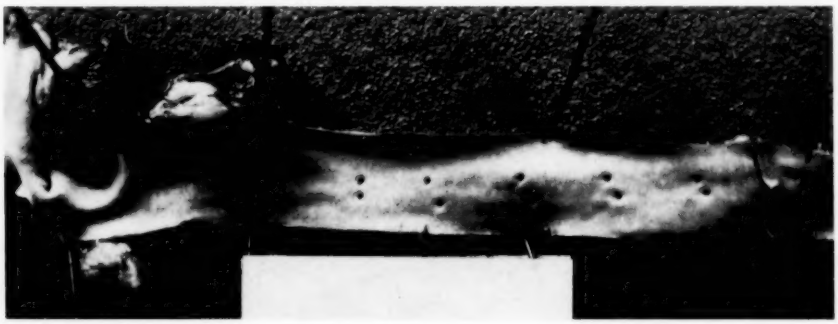

Fig. 1.—Normal rabbit aorta. (Gross photograph.)

RESULTS AND DISCUSSION

Infusion of synthetic L,D. alpha (dimyristoyl) lecithin into atherosclerotic rabbits resulted in a transient rise of blood cholesterol and a rise of lipid phosphorus (Table II). A gradual reduction of the hematocrit was noted after infusion.

At autopsy, the normal animals exhibited aortas which were completely free of arteriosclerotic change (Fig. 1). Histologic examination of these aortas

Fig. 2.

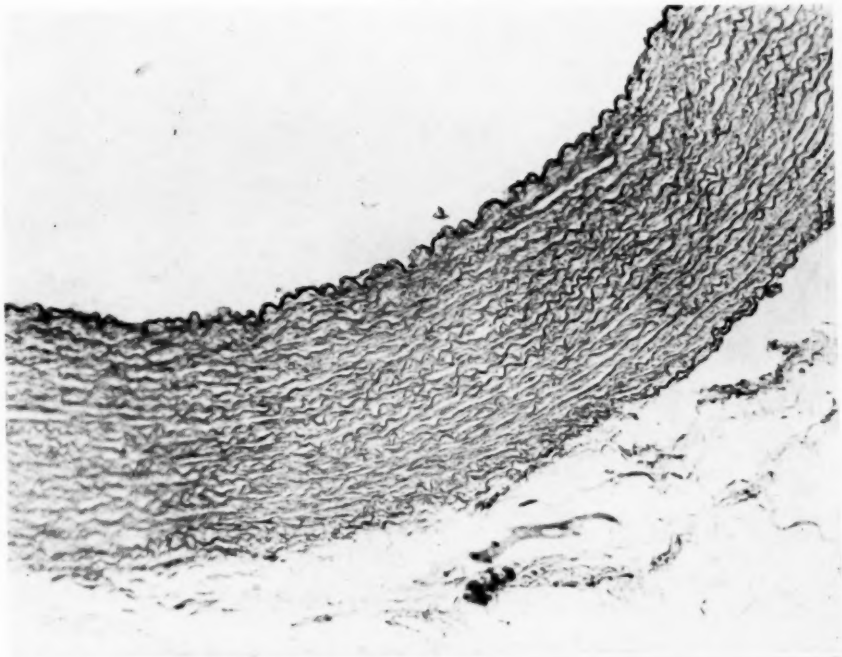

Fig. 3.

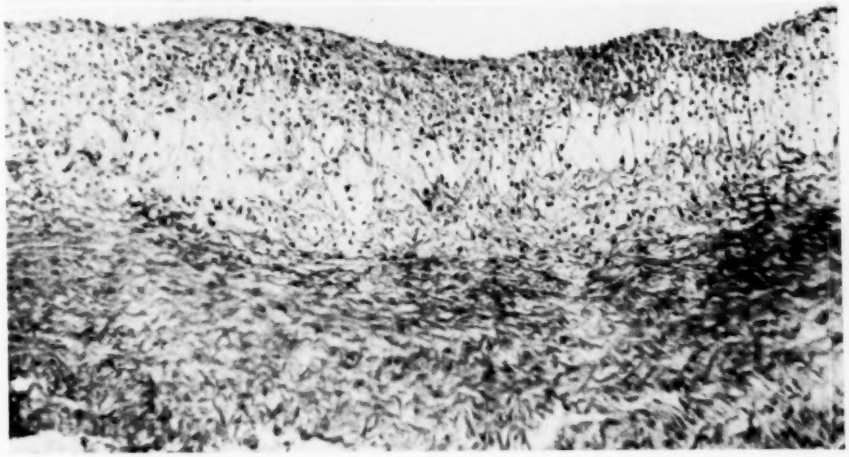

Fig. 2.—Normal rabbit aorta showing complete absence of atheromatous plaques. (Photomicrograph; magnification ×60.)

Fig. 3.—Rabbit aorta showing severe atheromatous plaque formation produced by high cholesterol diet. (Photomicrograph; magnification ×60.)

confirmed complete absence of arteriosclerotic change (Fig. 2). In the aortas in which atherosclerosis had been experimentally produced, atheromatous plaques were abundant and conspicuous. The plaques presented the customary yellow, elevated appearance and covered approximately 50 per cent of the intimal surface of the aortas. Their incidence was considerably greater in the

Fig. 4.—Aorta of atherosclerotic rabbit showing complete resolution of atheromatous plaques caused by 5 infusions of L,D. alpha lecithin. (Gross photo.)

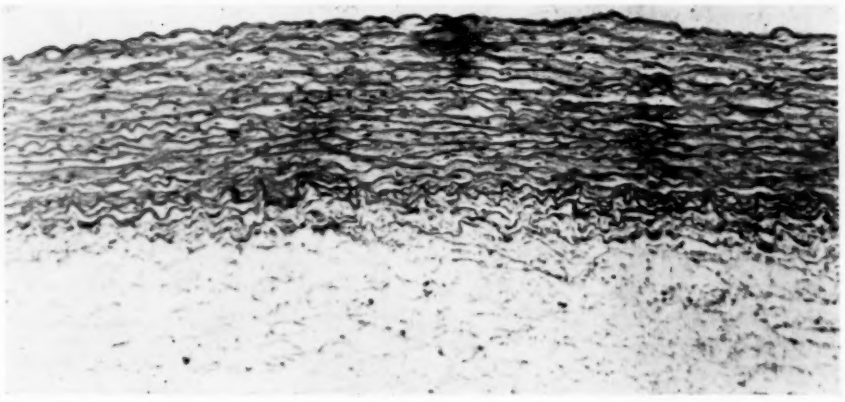

Fig. 5.—Rabbit aorta showing complete resolution of experimentally produced atheromatous plaques. This resolution was produced by 5 infusions of L,D. alpha lecithin. (Photomicrograph; magnification ×60.)

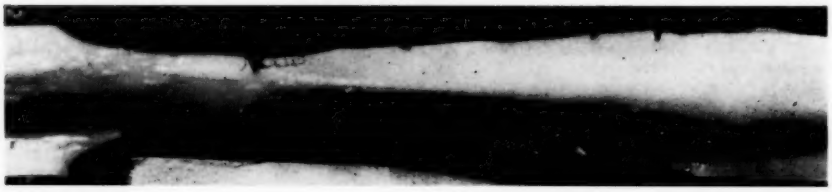

Fig. 6.—Rabbit aorta showing marked resolution of atheromatous plaques after infusion of L,D. alpha (dimyristoyl) lecithin. (Gross photo.)

arch and descending thoracic portions of the aortas than in the abdominal portion. In the arches the plaques covered nearly 90 per cent of the intimal surface. Microscopic sections of these aortas (Fig. 3) showed the thickness of the plaques to be one half to one third the total thickness of the aortic wall. The plaques were composed of numerous histiocytes possessing granular cytoplasm. In many instances these histiocytes extended into the deeper layers of the aortic wall.

The aorta of one animal in which experimental atherosclerosis had been produced, and which had received 5 infusions of L,D. alpha (dimyristoyl) lecithin, showed total absence of identifiable atheromatous plaques (Fig. 4). The intima was smooth and glistening. The vessel walls were elastic and did not appear to differ in any respect from the aortas found in normal animals. This complete resolution of atheromatous plaques was further substantiated by histologic examination (Fig. 5). A second sclerotic rabbit which also received 5 infusions showed very striking resolution of the plaques; however, a few still remained (Fig. 6).

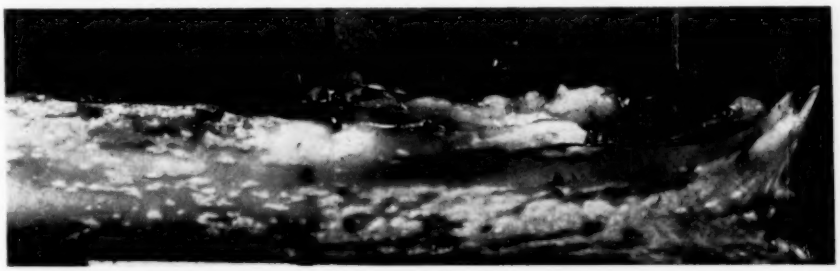

Fig. 7.—Rabbit aorta taken from killed control animal at the end of the experiment showing experimentally produced atheromatous plaques still present. This animal did not received lecithin infusions. (Gross photo.)

Because of technical difficulties, one rabbit received only 4 infusions and two rabbits received 3 infusions each. The aortas of these rabbits also showed distinct resolution of the atheromatous plaques; however, they did not return completely to normal. In these aortas the degree of resolution appeared to be roughly proportional to the amount of lecithin which had been infused. The total number of plaques was diminished. The color of the remaining plaques changed from yellow to white, and there was marked reduction in the total thickness of the remaining plaques as contrasted to the plaques in the untreated sclerotic aortas.

Two control rabbits, which had been rendered arteriosclerotic, were killed at the end of the experiment. These displayed very prominent atheromatous plaque formation (Fig. 7) with no indication of resolution. Thus, the resolution of atheromatous plaques was not merely the result of elapsed time.

These findings are in general agreement with those found by Friedman and his associates using a mixture of soybean phosphatide. The question remains: is lecithin the only active component in the mixture of naturally

occurring phosphatides capable of dissolving atheromatous plaques? The investigation of other members comprising the soybean phosphatide mixture is under way in this laboratory.

No striking difference was noticed between the cholesterol content of lecithin treated rabbit aortas and atherosclerotic control rabbit aortas (Table III).

TABLE III. EFFECT OF INFUSION OF L,D. ALPHA (DIMYRISTOYL) LECITHIN UPON CHOLESTEROL CONCENTRATION OF RABBIT AORTA

RABBIT (NO.)	INFUSIONS	WEIGHT OF DRY AORTA (MG.)	CHOLESTEROL (%)	LIPID PHOSPHORUS (%)
Atherosclerotic Rabbits (Untreated).—				
1		280	1.60	
2		375	3.40	0.086
3		382	4.10	0.066
4		288	6.49	0.099
5		360	6.54	0.063
Atherosclerotic Rabbits Treated With L,D. Alpha (Dimyristoyl) Lecithin.—				
6	3	210	5.40	
7	3	250	6.90	0.125
8	4	255	3.80	0.087
9	5	382	0.35	0.046
10	5	250	1.62	0.053
Normal Control.—				
11		112.5	0.85	
12		357	0.31	
13		257	0.33	
14		178	0.47	
15		900	0.47	

SUMMARY

The effect of intravenously infused synthetic L,D. alpha (dimyristoyl) lecithin was studied in the aortas of five rabbits with experimentally induced atherosclerosis.

A gradual increase in blood cholesterol concentration and a significant drop in hematocrit value followed the intravenous administration of the lecithin emulsion. These infusions appeared to effect a marked resolution of atheromatous plaques of rabbit aortas as compared with atherosclerotic control rabbits.

We wish to express our thanks to Mr. M. Kunstman for the preparation of L,D. alpha (dimyristoyl) lecithin.

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THE EFFECT OF ENDOTOXIN (SERRATIA MARCESCENS) UPON THE SYSTEMIC, PULMONARY, AND CORONARY HEMODYNAMICS AND METABOLISM OF THE INTACT DOG

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The coronary blood flow and myocardial extraction rates were studied in intact dogs during the shocklike state induced by endotoxin of Serratia marcescens. The hypotensive state was accompanied by changes in general metabolism, cardiac output and cardiac work, and circulatory dynamics equivalent to those observed in hemorrhagic shock. There was a decrease in coronary blood flow and cardiac efficiency associated with an increase in myocardial oxygen extraction.—ED.

THE EFFECT of endotoxin upon the animal and human has been studied for many years. This substance has been implicated in the "shocklike" state which results from the transfusion of contaminated blood,[1, 2] and has been shown to have an effect upon the growth of experimental tumors.[3] Most authors agree that depression of the blood pressure is a usual response, and is found in a wide variety of animals.[4] The subject has been reviewed in recent years by Burrows[5] and Thomas.[6]

Little information, however, appears in the literature concerning the effects of these substances upon the coronary circulation and myocardial metabolism of the intact animal. Therefore such a study, utilizing the endotoxin of *Serratia marcescens (Bacillus prodigiosus)* was designed; the results are now presented.

MATERIAL AND METHODS

Eight dogs, unselected by age or sex, and weighing between 15 to 25 kilograms, were used in this study. All appeared in good health at the time of study.

The animals were premedicated with morphine sulphate (3 mg. per kilogram) injected subcutaneously. This was followed in 1 hour by the intravenous injection of Dial-urethane†-Nembutal (50:50 mixture) in a dose of 0.25 ml. per kilogram.

Cardiac catheters were placed fluoroscopically in the right atrium, pulmonary artery, and coronary sinus. A Cournand needle was placed percutaneously in the femoral artery. Pressures were measured in these sites (except coronary sinus) by Statham strain gauges, and recorded on a "Poly-Viso." One hour after the intravenous anesthetic was given, cardiac

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†Courtesy of Ciba Pharmaceutical Products, Inc., Summit, N. J.

output was measured by the Fick principle and coronary blood flow by the nitrous oxide saturation method. The general method used and the reproducibility thereof have already been reported from this laboratory.[7, 8] Twenty minutes after completion of the control measurements, endotoxin (*S. marcescens*, Difco) was injected rapidly into the right atrium. The dose used was 1.5 mg. per kilogram. Cardiac output and coronary blood flow were remeasured within 5 minutes of the injection; the total "experimental" period was 25 minutes.

On completion of the experiment, the heart was removed, and the left coronary circulation was injected with T1824 or indigo-carmine dye; the weight of the stained heart was used to calculate cardiac efficiency by the accepted method. The animal's temperature was measured in the nasopharynx before and after the injection.

RESULTS

All figures in the tables are group means with standard deviations. Analysis was done by the Student "t" test, "significance" being accepted at the 5 per cent level. It is implicit that "statistical" and "physiologic" significance are not interchangeable terms.

The immediate effect of the endotoxin upon the pressures of the dog is exemplified in Fig. 1, which shows a rapid fall in mean femoral and right atrial pressures; an early rise in pulmonary arterial pressure is followed by a return to control level. The heart rate increases. These changes occurred in each dog, and persisted for at least 40 minutes after the injection—appreciably longer than the period of study proper.

The changes in general metabolism are presented in Table I. Minute respiratory exchange is maintained, but oxygen consumption and carbon dioxide production decrease. The "whole-body oxygen" extraction increases, and significant changes in carbon dioxide values are found.

The pressure work changes (Table II) show a decrease in cardiac output; cardiac acceleration then markedly reduces stroke volume. The pressure changes noted in Fig. 1 persist, so that left and right ventricular works decrease, but calculated peripheral and pulmonary resistances increase. Coronary hemodynamics and myocardial oxygen and carbon dioxide metabolism are shown in Table III, which demonstrates that coronary flow decreases in parallel

TABLE I. ENDOTOXIN

FACTOR	GENERAL METABOLISM	
	CONTROL	EXPERIMENTAL
Respiratory volume (L./min.)	2.7 ± 0.5	2.9 ± 0.7
Respiratory rate/min.	14 ± 5	16 ± 8
Tidal volume (ml.)	200 ± 71	203 ± 70
Oxygen consumption (ml./min.)	103 ± 18	68 ± 17*
Carbon dioxide production (ml./min.)	85 ± 17	61 ± 10*
Respiratory quotient	0.82 ± 0.04	0.90 ± 0.08*
Arterial oxygen content (vols. %)	16.7 ± 2.4	16.7 ± 2.3
Mixed venous oxygen content (vols. %)	12.7 ± 2.3	9.2 ± 3.4*
Δ Arterial-mixed venous oxygen (vols. %)	4.0 ± 0.8	7.5 ± 1.9*
Mixed venous carbon dioxide (vols. %)	54.2 ± 6.0	52.1 ± 7.0*
Arterial carbon dioxide (vols. %)	50.8 ± 6.6	46.0 ± 9.0*
Δ Mixed venous-arterial carbon dioxide	3.4 ± 1.2	6.0 ± 2.8*

Figures are means plus or minus standard deviations.
*p value 0.05 or less.

with the decrease in aortic pressure so that coronary vascular resistance remains substantially the same. Cardiac oxygen extraction increases as does cardiac carbon dioxide diffusion. However the changes cited, when compounded with the decrease in coronary blood flow, do not give a statistically significant decrease in cardiac metabolic rates for these two (O_2 and CO_2). The cardiac oxygen consumption directly determined from heart weight also fails to achieve a statistically significant change. There is a marked decrease in cardiac efficiency whether assessed directly from cardiac weight, or on an index basis.

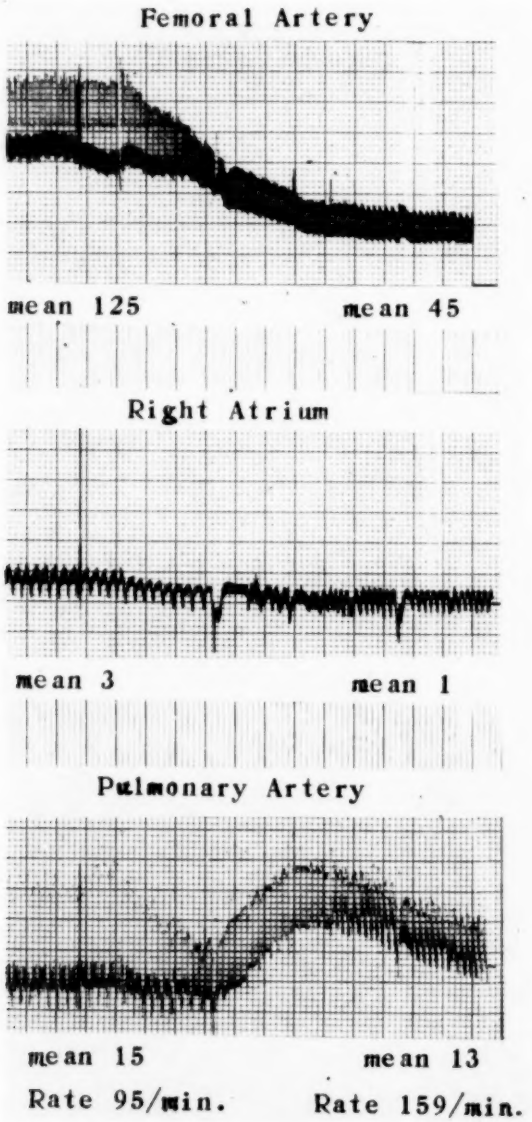

Fig. 1.—Immediate effects of injection of endotoxin, 1.5 mgm. per kilogram, into right atrium.

DISCUSSION

No variation in the animal's temperature was recorded, perhaps because of the relatively short period over which the experiment was conducted.[4] No evidence of tachypnea or increased minute volume of respiration occurred; this may be related to the maintenance of the body temperature at control values. The reduction in oxygen consumption and carbon dioxide production is evidence of interference with cellular metabolism, at least partly caused by the reduction in cardiac output as found in hemorrhagic shock.[9] The reduction in carbon dioxide production is relatively less than that of oxygen consumption, so that

TABLE II. ENDOTOXIN

FACTOR	PRESSURE AND WORK RESPONSES	
	CONTROL	EXPERIMENTAL
Cardiac output (L./min.)	2.6 ± 0.6	0.9 ± 0.4*
Heart rate/min.	81 ± 13	160 ± 26*
Stroke volume/ml.	32 ± 8	6 ± 4*
Right atrial pressure (mean, mm. Hg)	5 ± 1.6	2.5 ± 0.9*
Femoral blood pressure (mean, mm. Hg)	115 ± 10	69 ± 17*
Pulmonary arterial blood pressure (mean, mm. Hg)	15 ± 1.7	13 ± 3
Left ventricular work (Kg./M./min.)	4.0 ± 0.8	0.9 ± 0.5*
Stroke left ventricular work (Gm./M./min.)	49 ± 9	6 ± 5*
Right ventricular work (Kg./M./min.)	0.5 ± 0.1	0.1 ± 0.1*
Calculated peripheral resistance (dynes/cm.$^{-5}$/sec.)	3,552 ± 730	6,262 ± 1,800*
Calculated pulmonary resistance (dynes/cm.$^{-5}$/sec.)	507 ± 120	1,242 ± 380*

Figures are means plus or minus standard deviations.
*p value 0.05 or less.

TABLE III. ENDOTOXIN: CORONARY HEMODYNAMICS, CARDIAC O_2 AND CO_2 METABOLISM, AND CARDIAC EFFICIENCY

FACTOR	CONTROL	EXPERIMENTAL
Coronary blood flow (ml./100 Gm. myocardium/min.)	100 ± 26	52 ± 13*
Coronary flow/beat (ml./100 Gm./min.)	1.2 ± 0.2	0.3 ± 0.2*
Ratio, coronary flow/left ventricular work	25 ± 7	69 ± 31*
Coronary vascular resistance (units)	1.1 ± 0.3	1.3 ± 0.3
Coronary sinus oxygen (vols. %)	6.0 ± 1.4	3.1 ± 1.2*
Δ Arterial-coronary sinus oxygen (vols. %)	10.6 ± 2.4	15.0 ± 2.0*
Coronary sinus carbon dioxide (vols. %)	58.5 ± 7.0	51.8 ± 7.0*
Δ Coronary sinus-arterial carbon dioxide (vols. %)	8.8 ± 2.5	13.6 ± 2.2*
Cardiac respiratory quotient	0.82 ± 0.03	0.90 ± 0.07*
Cardiac metabolic rate-oxygen (c.c./100 Gm. heart/min.)	10.2 ± 1.8	7.7 ± 1.9†
Cardiac metabolic rate-carbon dioxide (ml./100 Gm. heart/min.)	8.4 ± 1.7	7.0 ± 1.6
Cardiac oxygen consumption‡ (ml./100 Gm./min.)	9.7 ± 2.4	7.2 ± 2.1†
Cardiac oxygen consumption/beat‡ (ml./100 Gm./min.)	1.2 ± 0.2	0.4 ± 0.1*
Index of efficiency (L. V. work/$CMRO_2$)	0.38 ± 0.08	0.12 ± 0.05*
Cardiac efficiency‡ (%)	20 ± 6	6 ± 3*

Figures are means plus or minus standard deviations.
*p value 0.05 or less.
†$p < 0.1 > 0.05$.
‡Derived from wet weight of left ventricle. Mean left ventricular weight 95 grams; range 76 to 109 grams.

respiratory quotient (exchange ratio) must increase. The increase in oxygen extraction closely parallels the findings in shock from other causes, e.g., hemorrhage.[9] The parallel extends to the pressure work responses; decrease in femoral mean pressure, cardiac output, and right atrial pressure and increase in heart rate are characteristic of hemorrhagic and other types of shock.[9, 10] The relatively greater decrease in cardiac output as compared to systemic and pulmonary blood pressure changes leads to an increase in the calculated resistances in the greater and lesser circulations. Similar results have been reported after injections of other types of toxin (e.g., those of the meningococcus[11] and *Clostridium oedematiens*[12]).

Although it is known that some bleeding into the gut occurs after the injection of endotoxin[6] autopsy in several of the animals studied revealed that this was of minor degree. Blood volume studies in dogs injected with meningococcal toxin showed little change.[11] It is likely, therefore, that hemorrhage played only a minor part in causing the aforementioned changes. It is likely that one of the primary causes is stagnation of the blood in the small vessels,[3, 9] much of which occurs in the splanchnic bed.[13] Most of the changes noted in Tables I and II are similar to those reported by other authors for different endotoxins, and are analogous to the phenomena which occur in shock from hemorrhage or other cause.[9, 10]

The decrease in coronary blood flow is profound, and associated with an increase in cardiac oxygen extraction. The resultant cardiac metabolic rate for oxygen shows a strong, but statistically insignificant ($p < 0.1 > 0.05$) trend to reduction. Similar changes in the cardiac metabolism of carbon dioxide appear. The increase in cardiac respiratory quotient parallels that of the whole animal. The marked decrease in left ventricular work is out of proportion to that of cardiac oxygen consumption, so that heart efficiency declines. Some of this latter change may be attributed to the concomitant tachycardia—of itself known to decrease cardiac efficiency.[7] The decrease in oxygen consumption per beat is parallel to the decrease in left ventricular stroke work, and to the change in coronary flow per beat. These changes closely resemble those found in similarly prepared dogs made hypotensive with mecamylamine,[14] or by the epidural injection of procaine.[15] With minor deviations, it is also the pattern of a study of coronary hemodynamics and myocardial metabolism in dogs suffering from hemorrhagic shock.[16] It has been said that coronary blood flow or cardiac oxygen usage will vary with cardiac output,[17] or with left ventricular work.[18] The factors mentioned all decreased, so that the correlation coefficients were significant, viz., cardiac output: coronary blood flow – R = 0.811; left ventricular work: cardiac oxygen consumption – R = 0.62. The association of heart rate times mean arterial blood pressure and cardiac oxygen consumption recently propounded[19] could not be fulfilled in this study, in which R = 0.08 for the two variables.

SUMMARY AND CONCLUSIONS

1. The endotoxin of *Serratia marcescens* causes a hypotensive state which is similar to that which follows the injection of other endotoxins.

2. This hypotensive state is equivalent to that which occurs in hemorrhagic shock, in terms of the changes in general metabolism, pressures, and heart work.

3. There is a decrease in coronary blood flow and cardiac efficiency associated with an increase in cardiac oxygen extraction. These changes are characteristic of those which follow profound hemorrhage.

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ALPHA$_2$-TYPE MULTIPLE MYELOMA

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A case of multiple myeloma is described in which the α_2-globulin represents 23 to 32 per cent of the serum proteins. Over half of the sternal bone marrow cells were myeloma cells with large, spongy, vacuole-containing cytoplasm, further characterized by an excentric localization of the nuclei. Some of the cells contained Russell bodies, and others contained multiple nuclei. In addition, the patient excreted 3.56 to 9.35 Gm. per day of paraprotein of the Bence Jones type. The urinary protein was isolated by dialysis and its properties were studied. The urinary paraprotein was quite homogeneous by electrophoresis and ultracentrifugation, and had a mobility of -4.6×10^{-5} cm.^{2} per second per volt and a sediment constant of 3.36 Svedberg units. The mobility of the urinary paraprotein by both paper and free electrophoresis was between the α_1- and α_2-globulins. The combination of an increased α_2-globulin in the serum and a urinary protein with the mobility of α_2-globulins is very rare. The clinical findings of this case are compared with those reported in the literature for cases of α_2-myelomatosis.

THE EXISTENCE of myelomatosis with elevated α_2-globulin has been questioned.[1] This may be due to the rarity of the abnormality and the difficulties in evaluating the specificity of the α_2-globulin increase. This component of the serum proteins has been found elevated in neoplasia, infections, and many other diseases. Owen and Rider[2] restricted the classification of the α_2-type myelomatosis to those cases in which α_2-globulin exceeded 20 per cent of the total serum protein. This may be used as a criterion in uncomplicated cases of myeloma. The paraprotein in multiple myeloma usually shows a characteristic narrow, homogeneous electrophoretic band. The immunochemical experiments of Morton and Deutsch[3] indicate that neither the serum nor the urine paraprotein is a normally occurring protein. They found that the urinary paraprotein in patients with multiple myeloma is more closely related to the serum paraprotein of the same patient than to the paraproteins of other patients or to normal gamma globulin.

Since Wuhrmann and coauthors[4] described the α_2-globulin myelomatosis, additional cases reported upon have been recently reviewed by Owen and Rider.[2] In these cases the α_2-myelomatosis was in no instance associated with α_2-type Bence Jones proteinuria. The case reported here has many interesting aspects, including the migration of the urinary paraprotein in the range of the α_2-globulins. The urine paraprotein was of the Bence Jones type. Some of its physical properties are described.

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MATERIAL AND METHODS

Case Report.—The patient, a 68-year-old white woman, entered the hospital in July, 1957, with a history of a 20 pound weight loss over a period of 6 months which was followed by an upper respiratory infection. She complained of increasing fatigue and melena on several occasions. On admission she was found to be chronically ill and extremely pale. The only abnormal clinical finding was a moderate hepatosplenomegaly. The blood pressure was 140/90 mm. Hg.

Laboratory tests revealed that she had a hypochromic, microcytic anemia with an erythrocyte count of 1.75 million and hemoglobin of 4.5 Gm. Leukocytes were normal in number and type. Other findings were as follows: blood urea nitrogen 36.5 mg. per cent; serum proteins 7.5 Gm. with a normal albumin : globulin ratio; urine specific gravity 1.008 with 3 plus protein; x-ray of the lower gastrointestinal tract showed diverticulosis; and upper gastrointestinal studies revealed a defect which suggested a duodenal ulcer.

One month later, a sternal marrow examination showed that over 50 per cent of the cells were myeloma cells. They were characterized by large, spongy, vacuole-containing cytoplasm and a few cells which contained Russell bodies. The cells were further characterized by an excentric localization of nuclei, and in some instances multiple nuclei were present (Fig. 1).

A survey of the bone structure showed generalized osteoporosis and a compression fracture of the fourth dorsal vertebra, but no definite lytic lesions could be found.

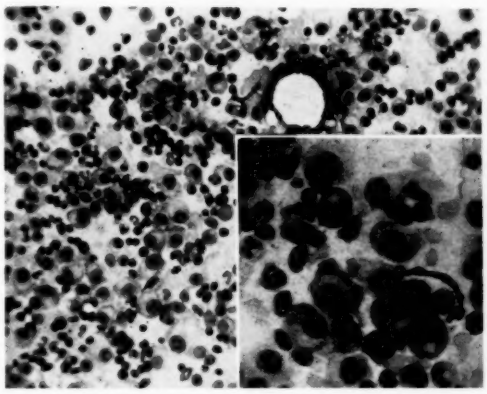

Fig. 1.—Photomicrograph of sternal bone marrow.

The serum contained 6.05 Gm. protein with a normal albumin : globulin ratio as determined by the precipitation of globulins with sodium sulfate. By paper electrophoresis the albumin was 45 per cent; α_1- 6 per cent, α_2- 23 per cent, β_1- 10 per cent, β_2- 9 per cent, and gamma globulin was 7 per cent of the total protein, giving an albumin : globulin ratio of 0.82. Other pertinent findings included: blood urea nitrogen 35 mg. per cent, serum calcium 5.1 mEq. per liter, serum phosphorus 4.2 mg. per cent, serum alkaline phosphatase 17 King-Armstrong units, and total serum cholesterol 365 to 400 mg. per cent. At this time the urine showed presence of urinary paraprotein, which will be described later.

The patient was again admitted to the hospital in September, 1957, about 2 months after the diagnosis was established. She showed severe renal failure, as evidenced by a blood nonprotein nitrogen of 142 mg. per cent, serum creatinine 18 mg. per cent, and CO_2 combining power 6.9 mEq. per liter. She also had considerable dyspnea, anemia, and widespread ecchymosis. The platelet count had dropped to 54,000. She died after generalized convulsions in a terminal deep comatose state. She had no evidence of hypertension at any time during her illness.

Isolation of the Urinary Paraprotein.—Six 24 hour urine samples were obtained from the patient at intervals over a 2 month period. The urine samples were preserved with thymol and used shortly after the end of the collection period. The urine was filtered through a Berkefeld N filter to remove cellular debris. Measured volumes were dialyzed 24 to 36 hours against running tap water in Visking 1⅝ inch cellophane tubing. The tubes were tied to a mechanical rocking device during the dialysis to give continuous agitation. The final volume was recorded and total and nonprotein nitrogen were determined. Samples were lyophilized and solutions were prepared from the pooled, dried material for the determination of the physical properties.

The dialyzed residues were analyzed for total nitrogen and nonprotein nitrogen, and the daily protein excretion was calculated from the nitrogen times 6.25.

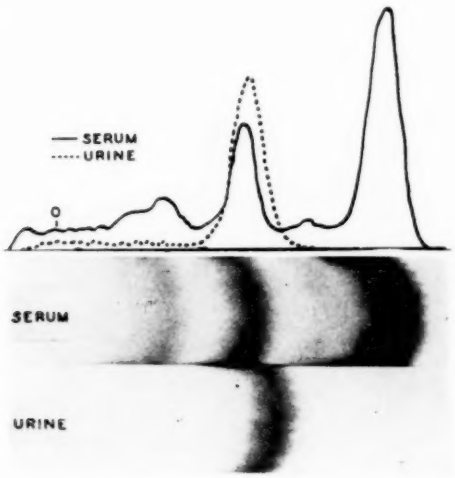

Fig. 2.—Paper electrophoresis of serum and urine proteins. Electrophoresis for 16 hours at 3° C. in acetate-NaCl-Veronal buffer, pH 8.6, ionic strength 0.05, horizontal table. The origin was at *o*.

RESULTS

The daily excretion of the protein varied between 3.56 and 9.35 Gm., average 5.5 Gm. The low methionine concentration found in Bence Jones protein by Putnam and Miyake[5, 6] prompted the administration of methionine to determine if this chemical might have an effect on the excretion of the paraprotein in the urine. The highest paraprotein excretion was observed 1 week after the patient had been given 3 Gm. methionine per day, orally. If the nitrogen analysis given by Schrade and co-workers[7] of Bence Jones proteins apply to this preparation, the total protein solids excreted would be 6 to 10 per cent higher. About 5 per cent of the nitrogen in the well-dialyzed urinary paraprotein was not precipitated by 10 per cent trichloroacetic acid.

Paper Electrophoresis.—The serum and urinary proteins were compared by paper electrophoresis. An LKB horizontal cell* was used and the separation was carried out at 3° C. in acetate-Veronal buffer, pH 8.6 and ionic strength 0.05. This method normally gives 2 β-globulins. The results of the scanning

*LKB-Produkter, Sweden.

of the paper electrophoretic strips of the serum and urine are superimposed in the upper part of Fig. 2. It will be noted that the paraprotein of the urine was about 95 per cent homogeneous and it migrated a little faster than the serum α_2-globulin. No albumin could be detected. The urinary paraprotein could be stained with ninhydrin. The total protein of the serum had a normal value of 6.05 to 7.5 Gm. per 100 ml. and in 10 serum samples analyzed 1 to 1.5 months before death the α_2-globulin represented 23 to 32 per cent of the total protein. A typical analysis as given in the clinical data shows an increase in α_2-globulin with a decrease in gamma globulin. A further analysis of the electrophoretic strips indicated that the mobility of the β-globulins of the serum is slower than found for normal serum, as the R_f of the β_1 and β_2 peaks is 36 and 30, respectively (normal is 40 and 33 by the method used).

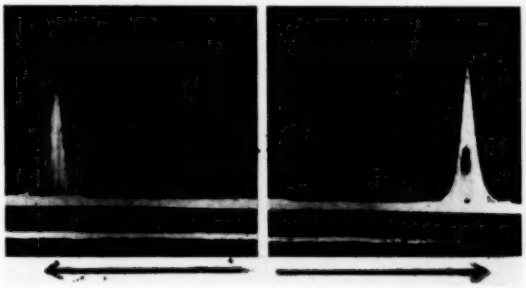

Fig. 3.—Electrophoresis of the urinary paraprotein. Photographed after 13,620 seconds' migration at 1° C. in Veronal buffer, pH 8.6, ionic strength 0.1, at a current of 20 Ma. Ascending boundary on the left and descending on the right. The photograph was supplied by Dr. Frank Putnam.

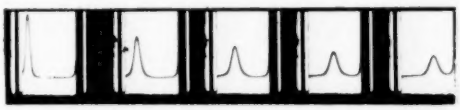

Fig. 4.—Sedimentation diagram of the urinary paraprotein. The analysis was in 0.1M sodium chloride; photographs were taken at 32 minute intervals at a rotor speed of 59,780 r.p.m. The photograph was furnished by Dr. Frank Putnam.

Electrophoretic and Ultracentrifugal Studies.—A sample of the lyophilized Bence Jones protein was submitted to Dr. Frank W. Putnam* who studied it by free electrophoresis and ultracentrifugation. Both methods showed the Bence Jones protein to be quite homogeneous. The results of the electrophoresis are shown in Fig. 3, from which the mobility of -4.6×10^{-5} cm.^{2} per second per volt was calculated—which is about midway between α_1- and α_2-globulins. In the ultracentrifuge (Fig. 4) the Bence Jones protein had a sedimentation constant of 3.36 Svedberg units. Putnam[6] pointed out that these values correspond closely to the value he reported for his protein-A.

Putnam found that the urinary protein acted as a typical Bence Jones protein. The optimum pH for precipitation in 0.1 ionic strength buffer on

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heating at 60° C. is 4.6, at which value 87 per cent of the protein precipitates. Practically no protein precipitated at pH 7. Putnam further submitted a sample of the Bence Jones protein to Dr. Leonhard Korngold, who tested it by the Ouchterlony agar gel diffusion method[5] and found it immunologically homogeneous but of the "atypical type A."

DISCUSSION

In most of the reports covering the α_2-type myelomas, no reference is made to the type of protein in the urine. The urine protein may be Bence Jones protein as a single entity or a mixture of normal and abnormal protein. Snapper and coauthors,[9] reviewing the urinary findings in 97 patients, showed that 90 per cent of them had albuminuria. Only 48 of 97 patients had Bence Jones proteinuria.

Snapper and co-workers[9] reported 3 cases of α_2-type myelomatosis. Two of the patients had Bence Jones proteinuria, but the characteristics of these urinary proteins were not given.

Sandkühler and Hugentobler[10] reported upon a number of cases with elevated α-globulins in the serum. A recalculation of their values shows only one patient with an α-globulin concentration of over 20 per cent and the Bence Jones protein migrated between the beta and gamma globulins. Their other patients with increased serum α-globulin also showed increases in other serum globulin fractions, but in no case was the paraprotein of the urine in α_2-globulin range.

Osserman and Lawlor[11] gave perhaps the most comprehensive report on the relation between the paraproteins in the serum and urine. In most cases, where the abnormal proteins were present in both fluids, the paraprotein in the urine migrated faster than it did in the serum. They found one case in which the abnormal urinary protein migrated as an α_2-globulin, but the serum showed a beta type myeloma protein. In the case of α_2-myeloma of Owen and Rider, the protein in the urine was in the gamma migration range. This patient also had the other components of serum in the urine.

In other reports of α_2-myeloma no reference to urinary proteins was given.[12-17] In no case that we found reported in the literature has an α_2–Bence Jones protein been associated with α_2-type serum pattern. Actually, the Bence Jones protein in our case moved a little faster than the α_2–serum globulin as is shown in Fig. 2 and calculated from the results shown in Fig. 3.

As Putnam pointed out, in our case the Bence Jones protein could be the abnormal myeloma protein in the serum. However, in no case has the metabolic relationship of Bence Jones protein to myeloma protein of serum been established. An excellent review by Putnam[18] suggests that Bence Jones protein is an abortive product of globulin synthesis. Accumulating evidence indicates Bence Jones proteins excreted by different patients are not identical.

A number of attempts have been made to correlate the type of α_2-paraprotein, the total serum protein concentration, the type of myeloma cells, and the prognosis. As the number of reported cases of elevated α_2-globulins in myeloma increases, exceptions to previous classifications are found. Owen

and Rider[2] discussed several of the exceptions. They suggest that their finding of a normal cholesterol value in their patient might be of value in the differential diagnosis between α_2-myelomatosis and the nephrotic syndrome. The high cholesterol level in the present case would not favor this suggestion.

SUMMARY

1. A case report of a patient with α_2-type myelomatosis is presented.

2. Bence Jones protein found in this case is relatively homogeneous. No albuminuria has been demonstrated. The Bence Jones protein was found to have a mobility of -4.6×10^{-5} cm.^{2} per second per volt, which is slightly faster than α_2-serum component. The sedimentation constant was 3.36 Svedberg units. Immunologically it appeared to be "atypical type A."

3. The relationship of myeloma protein to Bence Jones protein is discussed.

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SECRETION AND METABOLISM OF CORTISOL AFTER INJECTION OF ENDOTOXIN

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The adrenal secretory activity which follows the intravenous injection of endotoxins of gram-negative bacteria in pyrogenic and lethal doses was determined by the direct measurement of cortisol in the effluent of the cannulated lumboadrenal vein of the dog. Such injections produced a rapid increase in cortisol content, of the same order of magnitude as the adrenal secretory response to a large intravenous dose of exogenous corticotropin. The secretory response of the canine adrenal to exogenous corticotropin was diminished after the intravenous injection of a lethal dose of endotoxin. Hypophysectomy abolished the adrenal secretory response to injected endotoxin. Under the conditions of this study, intravenous injections of endotoxin did not produce adrenal secretory failure. Determination of biologic half-time of exogenous cortisol indicated delayed removal of cortisol from the plasma of dogs which received a lethal dose of endotoxin; but in dogs which received a pyrogenic dose of endotoxin the elevation of plasma cortisol appeared to be due solely to an increased adrenal secretion.—ED.

PERIPHERAL circulatory failure caused by endotoxin is frequently observed in patients who have infections caused by gram-negative bacteria.[1] An association of circulatory failure caused by infection with acute adrenal insufficiency has been postulated since the original reports of Waterhouse[2] and Friderichsen.[3] However, a deficiency of adrenocortical function has not been proved by the measurement of adrenal secretory products or their urinary metabolites. Data on 22 patients with severe infections complicated by circulatory collapse have been documented and in all of the patients the peripheral blood levels of cortisol were elevated above those of control subjects.[4] The significance of an elevated level of plasma cortisol cannot be assessed without information on the rate of removal of the steroid from the plasma or the rate of adrenal production. Urinary metabolites of cortisol may reflect adrenal secretion, but interpretations of these measurements are subject to wide error under conditions in which altered renal and hepatic functions are present.[5]

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The response to corticotropin as measured by changes in the concentration of plasma cortisol suggests continued integrity of adrenocortical function, but, for reasons already cited, does not indicate the magnitude of the response in patients in shock caused by infection. In an attempt to secure this information, cortisol in the adrenal venous blood of dogs was measured after the animals were subjected to endotoxin shock. The principal glycocorticoid of the canine adrenal cortex is cortisol.[6] Insertion of a cannula into the lumboadrenal vein of the dog and the collection of the total adrenal venous effluent provides a direct and specific method for the evaluation of adrenal secretory activity.

It has been established that a single injection of a large dose of endotoxin into mature experimental animals regularly results in fever, shock, and death.[7] Weil and associates[8] have shown that an intravenous injection of a lethal dose of endotoxin into the dog is followed by a predictable and reproducible characteristic sequence of events. Therefore, since the experimental conditions were reproducible and adrenal secretory activity could be measured directly, the assessment of the effect of endotoxins on adrenocortical function was carried out in the adult dog.

MATERIALS AND METHODS

Sixty-eight adult mongrel dogs ranging from 6 to 26 kilograms in weight were used in this study. All of the animals were anesthetized with pentobarbital sodium (30 mg. per kilogram) before they were subjected to experimental procedures. A permanent polyethylene cannula was placed in the right lumboadrenal vein of 40 of the dogs according to a technique described by Hume and Nelson.[9] This preparation permits intermittent collection of the total venous effluent of the right adrenal gland. Adrenal venous blood was collected for 5 minute periods at varying intervals; the cannula was filled with 4 per cent heparin contained in physiologic saline solution between the periods of collection. Experiments were carried out 18 to 24 hours after the cannula had been inserted. Peripheral blood specimens, obtained from the femoral vein by means of a polyethylene catheter of small gauge, were heparinized and the erythrocytes were separated from the plasma within 30 minutes. The samples were frozen and plasma cortisol levels were determined subsequently by a modification of the method of Silber and Porter,[10] as described by Wu and Mason.[11] This method is specific for steroids having the 17,21-dihydroxy-20-keto configuration which includes cortisol, cortisone, 11-deoxycortisol, and their A-ring reduced derivatives. Of these, only cortisol exists in the plasma in significant concentrations.[6]

Purified endotoxin of the Boivin type derived from *Eschirichia coli* and prepared by the method of Spink and Anderson[12] was administered to dogs in a dose of 1.0 mg. per kilogram. A single injection uniformly produced profound shock and eventually death. In 12 dogs a nonlethal dose (0.002 mg.) of a lipopolysaccharide derived from *Salmonella abortus equi* (Pyrexal) was administered intravenously; this did not produce shock but did cause a pyrogenic reaction.

EXPERIMENTAL PROCEDURE

1. After collection of a sample of adrenal venous blood, each of 7 dogs received a lethal dose of *E. coli* endotoxin injected intravenously. Adrenal venous effluent was collected at 30 and 120 minutes after the injection. The specimens were analyzed for cortisol and the output of cortisol from the right adrenal gland was then calculated by the formula: cortisol (micrograms per milliliter of plasma) times adrenal venous blood flow rate (milliliters per minute) divided by 2* equals output (micrograms per minute).

*The figure 2 is derived from the mean hematocrit value of 50 per cent.

After control specimens were obtained, an injection of 0.002 mg. of endotoxin (Pyrexal) was given to each of another 6 dogs, and subsequent samples of blood were collected from the adrenal vein at 5, 30, 60, 120, and 180 minutes. A rapid injection of 25 U.S.P. units of corticotropin* was given to each of 3 dogs after a control sample was obtained via the cannula in the adrenal vein. Specimens collected at 30, 60, 90, 120, and 180 minutes after the corticotropin injection were treated as aforementioned.

2. Ten dogs with adrenal vein cannulas were rapidly given an injection of 15 U.S.P. units of corticotropin after control specimens were obtained. Samples of blood from the adrenal vein were taken at 5, 10, and 120 minutes after the injection. Within the next 30 minutes a lethal dose of *E. coli* endotoxin was administered in a single injection, and blood was collected from the adrenal vein 5, 60, 120, and 180 minutes afterward. At 185 minutes after the administration of endotoxin a second injection of corticotropin was administered, and samples were taken at 5 and 10 minutes. This same procedure was followed in 7 dogs a few hours after they had been hypophysectomized transtemporally.[13] Another 7 dogs were given a constant infusion of corticotropin in physiologic saline solution at the rate of 0.3 U.S.P. units per minute. Specimens of blood from the adrenal vein were collected hourly for 3 hours; within the following 30 minutes a lethal dose of endotoxin was administered intravenously and samples were obtained at 5 minutes, and 1, 2, and 3 hours thereafter.

3. In order to determine the rate of removal of exogenous cortisol from the plasma, an intravenous injection of cortisol, as the succinate ester dissolved in 2 ml. of distilled water,† was given in a dosage of 5 mg. per kilogram (as cortisol) to 10 normal anesthetized dogs, to 6 dogs after an injection of a pyrogenic dose of endotoxin (0.002 mg.) had been given, and to 7 dogs who had received lethal doses of *E. coli* endotoxin (1.0 mg. per kilogram) intravenously. Samples of peripheral (femoral) venous blood were obtained immediately before the injection of the cortisol sodium succinate and at 30, 60, 90, 120, and in some, at 180 minutes thereafter. Plasma cortisol concentrations were determined as described. The sodium succinate ester of cortisol was used because it could be given in a small volume of diluent within a few seconds. Hydrolysis of the ester is complete within 15 minutes and the ester is not extracted from plasma by dichlormethane, which is the extraction solvent used in the determination of plasma cortisol. The disposal of this cortisol ester does not differ significantly from cortisol removal rates following infusions of cortisol-free alcohol in man.[4]

RESULTS

A. *Adrenal Secretory Response to Pyrogenic and to Lethal Doses of Endotoxin Compared With the Response to Exogenous Corticotropin.*—The mean outputs of cortisol from the right adrenal in response to these stimuli are shown on the graph in Fig. 1. The basal output of cortisol averaged less than 1 μg per minute. Thirty minutes after the injection of corticotropin, the output rose to a mean of 8.1 μg per minute with a range of 7.3 to 9.3 μg per minute. After this injection of corticotropin (25 U.S.P. units), the values remained elevated at 120 minutes with a mean output of 11.0 μg per minute, and a range of 7.5 to 13.2 μg per minute. In subsequent experiments in which smaller doses of exogenous corticotropin were used, maximal adrenal secretory responses were of shorter duration. In general, the duration of increased adrenal cortisol secretion was directly related to the dose of exogenous corticotropin. After a lethal dose of endotoxin, adrenal secretion of cortisol

*Sterile corticotropin injection, The Upjohn Company, Kalamazoo, Mich.
†Hydrocortisone sodium succinate, The Upjohn Company, Kalamazoo, Mich.

rose to an average of 8.9 μg per minute, with a range of 4.1 to 15.7 μg per minute; 120 minutes after the injection of endotoxin, cortisol output rose further to 9.6 μg per minute, with a range of 6.0 to 17.4 μg per minute. Thus the adrenal secretory response to a lethal dose of endotoxin was of the same order of magnitude as the response to a large dose of exogenous corticotropin.

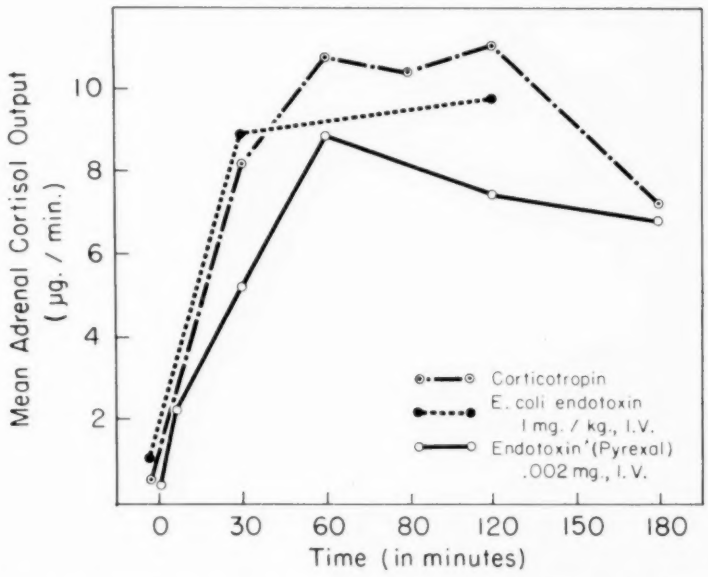

Fig. 1.—Changes in cortisol output from the right adrenal in the dog after the intravenous administration of pyrogenic and lethal doses of bacterial endotoxin and after a single injection of corticotropin, 25 U.S.P. units.

The injection of a pyrogenic dose of endotoxin, which resulted in neither hypotension nor death, increased the output of cortisol, but to a lesser extent than did a lethal dose of endotoxin or exogenous corticotropin. The average maximum value of 8.9 μg per minute (range 4.0 to 11.2 μg per minute) was achieved within 60 minutes after injection; both the rise and the decline in cortisol output were more gradual. Five minutes after the injection of the pyrogen, significant increases in cortisol secretion occurred in only 2 of the 6 animals tested, but by 30 minutes 4 of the dogs demonstrated a rise in cortisol output. During this period the rectal temperature increased an average of 0.6° C., but correlation between cortisol output and fever was erratic, and in 2 dogs large increments in cortisol production *preceded* changes in rectal temperature. It had been shown previously that the response of adrenal secretion to endotoxin is not due to fever per se, since the establishment of tolerance to the pyrogenic action of endotoxin did not influence the accelerated secretory response of the adrenal to endotoxin.[14]

B. *The Influence of Lethal Doses of Endotoxin on the Response of Adrenal Secretion to Exogenous Corticotropin in Intact and in Hypophysectomized Dogs.*—

1. *Intact animals:* These data are represented graphically in Figs. 2 and 3. In the intact animal the response to the same dose of corticotropin 5 minutes after injection varied from 4.0 to 17.3 μg per minute with an average cortisol output of 9.5 μg per minute. Two collections at 5 and 10 minutes after injection of corticotropin were found to encompass maximal responses in most of the animals tested. The cortisol output fell toward the base line levels after 2 hours to a mean value of 4.4 μg per minute (range, 1.0 to 10.8 μg per minute). It should be recalled that in dogs given a larger dose of corticotropin (25 U.S.P. units) in the initial experiments the output of cortisol continued at a maximum throughout this period. A longer interval between the administration of corticotropin and the injection of endotoxin would have been necessary had the dose of corticotropin not been reduced to 15 units.

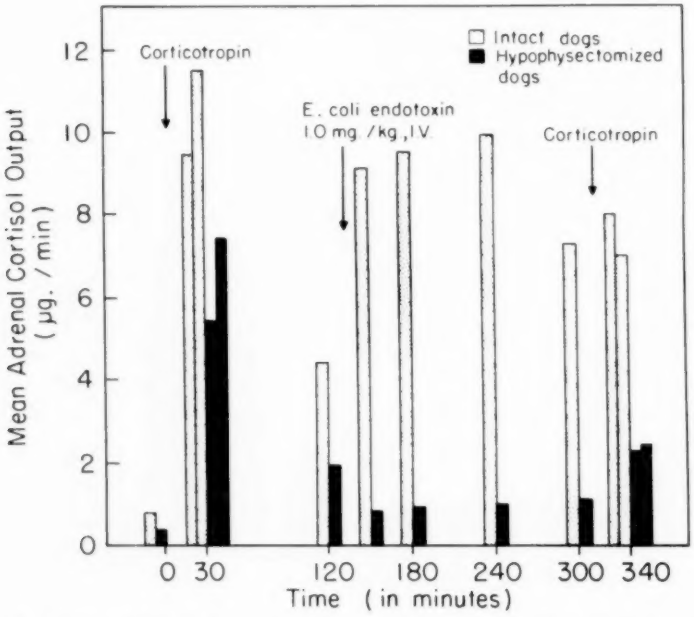

Fig. 2.—Influence of lethal doses of *E. coli* endotoxin on canine adrenal secretion of cortisol and the adrenal secretory responsiveness to exogenous corticotropin.

In general, the response to a lethal dose of endotoxin was of the same order of magnitude as that for corticotropin. Thus, 5 minutes after the injection of endotoxin, when the dogs were hypotensive and hyperpneic, the mean cortisol output was 9.1 μg per minute (range, 2.4 to 28.3 μg per minute). Three hours after this injection the dogs were hypotensive and febrile, but cortisol output had declined to an average of 7.3 μg per minute (range, 2.3 to 12.0 μg per minute). Two of the 10 animals studied died by the end of this period.

In one dog the cortisol output from the right adrenal gland was 5.3 μg per minute 5 minutes before death. In another animal which died within 10 minutes after the collection of the 2 hour specimen, this value was 6.8 μg per minute. In the surviving 8 animals, corticotropin produced a small but definite rise in adrenal cortisol output. This increment was shortlived, lasting only 5 minutes. It is evident from these experiments, that although the adrenal secretion of cortisol was well above base line values at the end of 3 hours after the injection of a lethal dose of endotoxin, there was an attenuation of response to exogenous corticotropin.

2. *Hypophysectomized animals:* Further definition of the degree of change in adrenal secretory activity in response to corticotropin after endotoxin shock was attempted in experiments with hypophysectomized dogs. Studies on the hypophysectomized dog were carried out within 3 to 6 hours after hypophysectomy. Hypothermia was prevented by warming the animals with incandescent lamps during the procedure. The response of the hypophysectomized dog to corticotropin was less pronounced than that of the intact dog. A maximum mean output of cortisol of 7.5 μg per minute (range, 3.2 to 17.2 μg per minute) was reached 10 minutes after the injection of corticotropin.

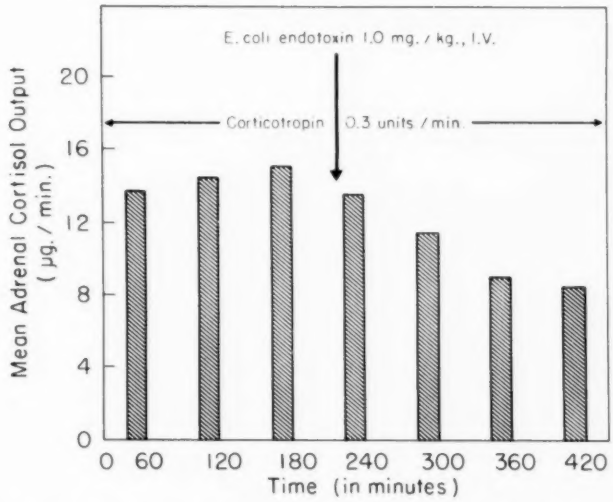

Fig. 3.—The effect of a lethal injection of *E. coli* endotoxin on the output of cortisol from the right adrenal gland during a constant infusion of corticotropin (0.3 units per minute).

Cortisol output did not rise within 3 hours after the injection of endotoxin. On the basis of these data, endotoxin does not have a direct stimulatory effect on adrenal secretory activity. The magnitude of response to corticotropin after endotoxin was diminished in the hypophysectomized anmials, the average maximum response being 2.4 μg per minute (range, 1.0 to 3.7 μg per minute).

3. *Constant infusion of corticotropin in intact animals:* The effect of endotoxin on adrenal cortisol output after an initial response to exogenous

TABLE I. MEAN PLASMA BIOLOGIC HALF-TIMES OF INJECTED CORTISOL AND MEAN PLASMA CORTISOL LEVELS AFTER THE INTRAVENOUS ADMINISTRATION OF 5 MG. CORTISOL PER KILOGRAM (AS THE SUCCINATE ESTER) IN DOGS GIVEN PYROGENIC AND LETHAL DOSES OF ENDOTOXIN

SUBSTANCES ADMINISTERED	NO. OF ANIMALS	MEAN PLASMA CORTISOL (μG/100 MG.)						MEAN BIOLOGIC HALF-TIME OF CORTISOL (MIN.)
		BEFORE EXOGENOUS CORTISOL	MIN. AFTER THE INJECTION OF CORTISOL 21-HEMISUCCINATE					
			30	60	90	120	150	180	
Controls	10	7 (0-12)	128 (100-193)	91 (75-146)	66 (41-126)	45 (25-91)			56.6
Endotoxin (Pyrexal, 0.002 mg. I.V.)	6	26 (16-45)	111 (100-127)	52 (47-60)		25 (4-33)		13 (0-27)	40.1
Endotoxin (*E. coli*, 1.0 mg/Kg. I.V.)	7	28 (5-52)	140 (75-168)	106 (70-152)	86 (57-146)	65* (34-110)			116.6
Endotoxin (*E. coli*,† 1.0 mg/Kg. I.V.)	5			46 (19-72)		52 (18-101)		53 (20-98)	

*One animal had died at this time and the data are computed on the basis of 6 determinations.

†No exogenous cortisol administered, mean levels are tabulated in minutes after endotoxin.

corticotropin was also assessed by means of a prolonged constant infusion of large amounts of corticotropin. The data are represented graphically in Fig. 3. The infusion of corticotropin at a rate of 0.3 units per minute was continued for 7 hours. By the end of the third hour of the infusion the cortisol output averaged 15.4 μg per minute. A lethal dose of endotoxin was injected into the dog during the infusion of corticotropin. One hour after the endotoxin injection the output of cortisol dropped to 11.3 μg per minute even though the infusion of corticotropin was continued. Adrenal cortisol output fell progressively, and within 3 hours after the endotoxin had been given it reached an average of 8.3 μg per minute. One animal died immediately following collection of a specimen 1 hour after the injection of endotoxin. The cortisol secretion of the dog was 10.1 μg per minute just prior to death. Another dog succumbed 30 minutes following the collection of a specimen 2 hours after the administration of endotoxin, and the cortisol output just before death was 3.6 μg per minute.

Lethal doses of endotoxin markedly stimulate adrenal secretion of cortisol but simultaneously the secretory response of the adrenal to exogenous corticotropin is progressively diminished. These findings are in contrast to the reported results in hemorrhagic shock in dogs in which the adrenal secretory response to exogenous corticotropin was actually found to be enhanced.[9] In hemorrhagic shock, only when the mean blood pressure was reduced below 35 mm. Hg was there reduction in cortisol output. Mean femoral arterial pressures were determined in several dogs after administration of lethal doses of endotoxin and, except for an initial sharp decline, there was partial recovery of the mean arterial pressure to levels well above 35 mm. Hg in all animals examined for a period exceeding the duration of the experiments. Weil and co-workers[5] reported arterial pressures exceeding 50 mm. Hg in the dog for a 5 hour period after the injection of endotoxin in a dosage up to 5 times that used in the experiments reported here. Pyrogenic doses of endotoxin that did not produce hypotension or death did not attenuate the adrenal secretory response to corticotropin in studies reported elsewhere.[14]

C. *Concentration of Cortisol in the Peripheral Venous Plasma in Dogs After Injections of Pyrogenic or Lethal Doses of Bacterial Endotoxins.*—These data are given in Table I. Thirty minutes after the injection of either pyrogenic or lethal doses of endotoxin plasma cortisol levels rose significantly. No appreciable difference was observed in either group of dogs, which suggests that the main independent variable affecting plasma cortisol concentration is increased adrenal secretory activity.

D. *The Rate of Removal of Cortisol From the Plasma When Infused as the Succinate Ester.*—Levels of plasma cortisol after the rapid infusion of cortisol sodium succinate in a dosage of 5 mg. per kilogram (as cortisol free alcohol) are shown in Table I. Although the elevated plasma cortisol levels in the dogs which received a pyrogenic dose of endotoxin and in those which received a lethal dose were not dissimilar, following the infusion of cortisol the 30 minute levels in the animals after a lethal dose of endotoxin were

significantly higher than in those which received a pyrogenic dose. Levels of cortisol in the plasma after the infusion of cortisol 21-hemisuccinate are plotted on semilogarithmic paper at different time intervals. These data are presented graphically in Fig. 4. The initial preinfusion or endogenous values are not subtracted from subsequent postinfusion levels. The levels obtained are the result of at least 2 variables: the level of adrenal secretory activity and the rate of removal of cortisol from the plasma. In experiments described earlier in this report it was shown that adrenal secretory activity was accelerated but that it diminished progressively after a lethal dose of endotoxin. Since the level in the peripheral plasma is relatively constant and adrenal secretion of cortisol is gradually diminishing during the period of this experiment, one possible explanation is that the rate of removal must also be reduced. An infusion of cortisol removes a significant endogenous contribution by inhibition of cortisol secretion and, thus, direct measurements of the rate of removal of plasma cortisol more accurately mirror its disposal.

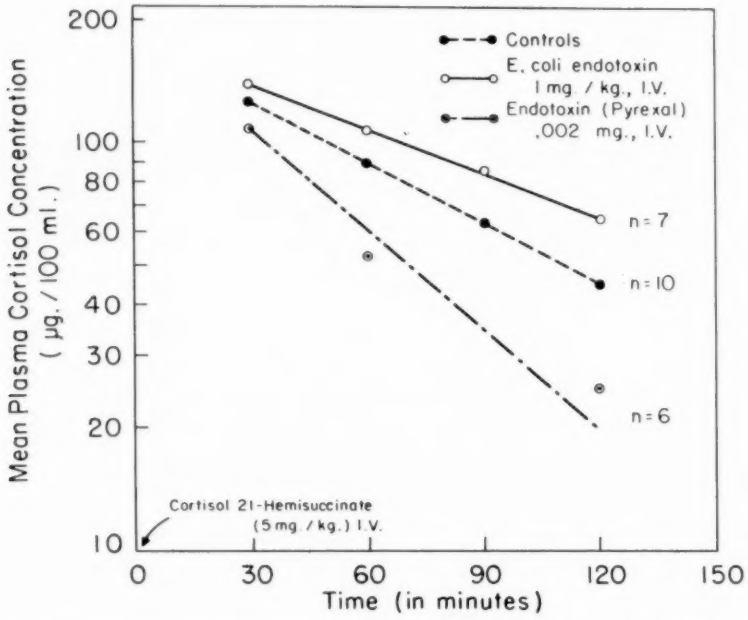

Fig. 4.—Influence of pyrogenic and lethal doses of bacterial endotoxins on the removal rate of exogenous cortisol injected as the succinate ester.

The mean biologic half-time of cortisol in the plasma calculated from the plasma levels after an infusion of cortisol by the method of the least squares may then be used as a rough index of the metabolic disposal of cortisol. This half-time value was 56.6 minutes in the control animals, 40.1 minutes in dogs given only pyrogenic doses of endotoxin, and 116.6 minutes in dogs given lethal doses of endotoxin. The half-time value for cortisol in the plasma is determined to a large extent by the functional integrity of the liver and the

blood flow to the organ.[5] The intense congestion and stasis of blood in the livers of dogs after administration of lethal doses of endotoxin have been described.[8] Thus, elevated levels of cortisol in the plasma of dogs after the production of endotoxin shock may be due in large part to impaired disposal of cortisol.

COMMENT

In the foregoing studies, no secretory failure of the adrenal glands could be demonstrated in dogs after the injection of pyrogenic or lethal doses of bacterial endotoxins. On the other hand, increased secretion of the adrenal after administration of endotoxin was demonstrated in all animals tested. These observations are in accord with the studies of Jones and co-workers[15] in the guinea pig and Wexler and associates[16] in the rat. The finding of increased cortisol production after administration of a lethal dose of endotoxin in the dog is in accord with the observations made in human beings. In patients in shock caused by infection the level of cortisol in the plasma was invariably elevated. In nearly half of the patients the metabolic disposal of exogenous cortisol continued at a normal rate which suggested an acceleration of cortisol production. In those patients who died, however, the rate of removal of exogenous cortisol from the plasma was retarded and for this reason it was not possible to interpret the exact significance of the increased concentration of endogenous cortisol in the plasma. The similarities between the results of the experiments reported here and the observations in human patients are striking. There was a normal rate of removal of cortisol from the plasma of dogs given pyrogenic doses of endotoxin but, in those dogs given a lethal dose of endotoxin, extremely slow removal rates were observed. However, both experimental groups produced supranormal amounts of cortisol in the adrenal venous effluent. The relative contribution of secreted cortisol to the high circulating levels of the hormone was greater in the animals that received a nonlethal dose of endotoxin than in those that were given a lethal dose. These in vivo experiments differ from the observations of Rosenfeld[17] on the in vitro effects of bacterial pyrogens on corticosteroidogenesis of isolated perfused calf adrenals. He reported certain bacterial lipopolysaccharides had an inhibitory effect on corticosteroid synthesis.

One significant observation in the studies reported here is the diminished response of the adrenals to corticotropin following a lethal dose of endotoxin. Rich[18] has described degenerative lesions in the zona fasciculata of the adrenal cortex in humans dying of circulatory collapse due to bacterial infection. He has reproduced these changes in the adrenal cortex of the rat following large doses of corticotropin, and has suggested that perhaps such histological change originates from prolonged intensive stimulation of the adrenals by endogenous corticotropin in patients with severe infection. Significant changes in the adrenal cortices of the dogs used in these studies could not be demonstrated grossly or microscopically. The attenuated response of the adrenals to exogenous corticotropin in terms of cortisol production cannot be explained by an adverse

effect of corticotropin per se since the hypophysectomized dog showed the same diminution in adrenal secretory response to corticotropin as did the intact animal.

Decreased responsiveness to corticotropin after an injection of a lethal dose of endotoxin occurs progressively and is significant at 2 to 3 hours following the establishment of endotoxin shock. At this stage, Weil and co-workers[8] have suggested that peripheral vasodilation and pooling of blood in post-capillary vessels occur. This peripheral stasis of blood leads inevitably to anoxia of the perfused tissues. It has been shown that the biosynthesis of cortisol can proceed anaerobically through the formation of progesterone but that 3 sequential hydroxylations cannot.[19] Molecular oxygen is required for the reactions catalyzed by 17 α-hydroxylase, 11 β-hydroxylase, and 21-hydroxylase; a-hydroxylation at carbon 17 is the most sensitive to oxygen lack followed by 11 β-hydroxylation.

Protection against the lethal effect of bacterial endotoxins by pretreatment with cortisol or its analogs has been demonstrated in various mammalian species.[7, 12, 20] Protection was afforded by doses of steroid hormones far in excess of the amount the particular experimental animal was capable of producing endogenously. Any suggestion that beneficial effects of adrenocortical substances in a given situation provide evidence of adrenal insufficiency is unwarranted. It has not been possible to demonstrate secretory failure of the adrenal glands in the dog after lethal doses of endotoxin producing microorganisms.[4] On the other hand, the demonstration that adrenocortical function is not disordered after a lethal dose of endotoxin does not preclude the use of cortisol or its analogs as therapeutic agents. In studies reported in detail elsewhere,[20] the serum glutamic oxalacetic transaminase activity was determined in a group of dogs injected intravenously with endotoxin prepared from *E. coli*. There was a prompt rise in transaminase and plasma concentration of cortisol. It has been generally assumed that elevations in serum transaminase are associated with cellular injury. It was apparent that maximal endogenous secretion of cortisol did not prevent the rise in transaminase levels. However, when large doses of cortisol (10 to 20 mg. per kilogram) were given with the endotoxin there was only a small rise in the levels of transaminase. All of the dogs which received no steroid died within 18 hours, whereas 60 per cent of the animals which were treated with cortisol survived. Ebert and colleagues[22] were unable to obtain protection by cortisone against the hypotensive effects of meningococcal endotoxin. This is an agreement with the experience of this laboratory.

SUMMARY

1. The intravenous injection of endotoxins of gram-negative bacteria in pyrogenic and lethal doses produces a rapid increase in adrenal secretory activity, determined by the direct measurement of cortisol in the effluent of the cannulated right lumboadrenal vein of the dog. This response to endotoxin is of the same order of magnitude as the adrenal secretory response to a large intravenous dose of exogenous corticotropin.

2. The secretory response of the canine adrenal to exogenous corticotropin is diminished after the intravenous injection of a lethal dose of endotoxin. The attenuation of adrenal responsiveness to exogenous corticotropin by endotoxin was demonstrated by intermittent and constant infusions of corticotropin. The significance of this finding is discussed.

3. Increased adrenal secretion of cortisol in response to injected endotoxin is abolished in hypophysectomized dogs.

4. The concentration of cortisol in the peripheral venous plasma rose significantly in all animals given either pyrogenic or lethal doses of endotoxin intravenously.

5. The biologic half-time of exogenous cortisol in the plasma of normal anesthetized dogs was 56.6 minutes; in dogs given a pyrogenic dose of endotoxin, 40.1 minutes; and in dogs given a lethal dose of endotoxin, 116.6 minutes. Therefore, the elevated levels of cortisol in the dogs which received a pyrogenic dose of endotoxin is due solely to increased adrenal secretion of cortisol; in the dogs which received a lethal dose of endotoxin, increased concentrations of cortisol in the plasma are due in part to increased adrenal secretion of cortisol and in part to retarded removal of cortisol from the plasma.

6. Endotoxins of gram-negative bacteria injected intravenously into dogs did not produce adrenal secretory failure.

We wish to acknowledge the valuable advice of Drs. H. L. Mason and R. H. Silber in the technique of cortisol analysis.

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COMPARISON OF METABOLIC EFFECTS OF HIGH AND MASSIVE DOSES OF PREDNISONE

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The occasional clinical requirements for excessively high doses of corticoids in the treatment of patients with diseases such as systemic lupus erythematosus and pemphigus prompted a comparison of the metabolic effects of high and massive doses of prednisone. Studies of metabolic balance were made on three patients while they were receiving 100 mg. and 1,000 mg. of prednisone daily. In one subject the loss of nitrogen was not increased as a result of the increased dose of prednisone. The catabolic effect of 1,000 mg. of prednisone was greater than that of 100 mg. in the other 2 patients, although even these effects were not marked. In the one patient with leukemia, the higher dose of prednisone increased the loss of lymphoid tissue nitrogen by 1.6 Gm. daily. Moderate sodium retention was noted at the 1,000 mg. dose level.

These data suggest that 100 mg. of prednisone a day can produce a nearly maximal catabolic effect. This finding does not provide any basis for understanding the occasional therapeutic benefits of massive doses of corticoids.

UNUSUALLY high doses of cortisone may be required for the treatment of patients with certain life threatening diseases. Patients with systemic lupus erythematosus may, during periods of crisis, need amounts of cortisone in excess of 300 mg. a day in order to achieve a therapeutic effect.[1] The treatment of patients with pemphigus[2, 3] similarly includes massive doses of these compounds. More recently it has been suggested that better therapeutic effects may be noted in patients with acute leukemia and certain lymphomas with massive doses of prednisone[4] but this has not been substantiated.[5]

Because of these clinical observations, it seemed appropriate to examine and compare the metabolic changes which result from high and massive doses of prednisone. It was also of theoretic significance to determine whether increasing catabolic effects could be obtained by increases of the dose of corticoids beyond the level usually considered high. Cortisone and prednisone differ qualitatively only in their effect in causing sodium retention. There are, however, few quantitative data bearing on this point.[6-9] It has been generally agreed that sodium retention is not noted with moderate doses of prednisone. However, in patients receiving high levels of this compound edema has been noted on several occasions.[8, 10] The studies presented here document the occurrence of sodium

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TABLE I. METABOLIC BALANCE DATA OF THE EFFECTS OF 100 MG. AND 1,000 MG. PER DAY OF PREDNISONE

PATIENT	DAYS	DAILY DOSE	Ca (MG.)	P (MG.)	N (GM.)	P_{Ca}* (MG.)	P_N† (MG.)	$P_{Ca} + P_N$ (MG.)	P'‡ (MG.)	Na (mEq.)	K (mEq.)	Cl (mEq.)	17 OHS§ (MG./24 HR.)
A. C.	1- 6	Cortisone acetate 37.5 mg.	− 291	− 99	+ 9.6	− 145	+ 640	+ 493	− 593	+171	+ 26	+ 99	12.5
Breast cancer; hypophysectomy	7-12	Prednisone 100 mg.	− 263	−1,523	−21.2	− 131	−1,415	−1,546	+ 23	−231	− 57	−250	32.7
	13-18	Prednisone 15 mg.	− 155	+ 356	− 1.9	− 77	− 127	− 49	+ 405	− 7	+ 61	− 31	3.7
	19-24	Prednisone 15 mg.	+ 517	+ 680	+ 3.6	+ 258	+ 240	+ 498	+ 181	+105	+ 25	+172	3.7
	25-30	Prednisone 1,000 mg.	− 443	−1,033	−19.9	− 221	−1,325	−1,546	+ 513	+228	−135	+252	233.0
M. D.	1- 6	Prednisone 15 mg.	+ 555	+ 252	+ 4.5	+ 277	+ 300	+ 577	− 325	− 6	+ 9	− 30	
Breast cancer	7-12	Prednisone 100 mg.	+ 648	− 492	− 3.0	+ 324	− 200	+ 124	− 616	+ 52	− 38	+ 62	22.1
	13-18	Prednisone 15 mg.	+ 666	+ 65	+ 0.0	+ 333	+ 40	+ 373	− 308	+ 54	+ 53	+ 94	2.7
	19-24	Prednisone 15 mg.	+ 947	+ 765	+13.4	+ 473	+ 892	+1,365	− 601	+133	+ 73	+141	2.7
	25-30	Prednisone 1,000 mg.	+ 196	−2,009	−12.5	+ 98	− 831	− 733	−1,276	+210	− 70	+129	198.0
	31-36	Prednisone 15 mg.	+ 506	+ 326	+14.9	+ 253	+ 993	+1,246	− 920	−144	+129	−162	8.4
S. Y.	1- 6	Cortisone 50 mg.	− 237	+ 695	+15.5	− 118				+ 13	+ 78	+ 48	
Chronic lymphatic leukemia	7-12	Prednisone 100 mg.	+ 192	−3,240	−23.1	+ 96				−208	− 64	−333	24.3
	13-18	Prednisone 15 mg.	+ 390	+1,022	+ 6.3	+ 195				+ 80	+ 84	+ 43	2.8
	19-24	Prednisone 15 mg.	+ 530	+ 987	+ 8.4	+ 265				+162	+ 40	+ 87	2.8
	25-30	Prednisone 1,000 mg.	+ 140	−5,497	−48.1	+ 70				+321	−257	+308	161.2
	31-36	Prednisone 15 mg.	−2,478	+ 802	+ 1.5	−1,239				−123	+ 98	−123	6.1

Figures in all the columns except the last represent total balance for 6 day periods. For patient S. Y., the part of the phosphorus balance not caused by nitrogen was used to calculate tumor and muscle nitrogen. (See text, and Table II.)

*Theoretic phosphorus balance caused by changes of calcium balance.

†Theoretic phosphorus balance caused by changes of nitrogen balance.

‡Phosphorus not moving with calcium or nitrogen.

§Average daily value.

retention with prednisone and thus suggest that prednisone differs quantitatively rather than qualitatively from cortisone, although this difference is marked.

METHODS

Three patients in the Metabolic Ward of the James Ewing Hospital were studied. They were fed from the Metabolic Diet Kitchen and received the same diet throughout each study. Sample diets were analyzed at the onset of each study and every 3 weeks; leftover food was analyzed. Activity was unrestricted. Caloric intake was adequate in all patients. The methods of analysis of sodium, potassium, calcium, phosphorus, nitrogen, uric acid, CO_2 content, and chloride have been previously reported.[11] Urine corticoids were determined by a modification of the method of Silber and Porter.[12]

Prednisone was administered at dosages of 100 and 1,000 mg. a day in divided doses. Each level of prednisone was studied for 6 days and the study periods were separated by pre- and postcontrol periods.

Balance data have been plotted by the method of Reifenstein and co-workers.[13] The theoretic phosphorus balances were taken as the sum of the phosphorus balance caused by changes in calcium and protein, using values of 500 mg. of phosphorus per gram of calcium and 67 mg. of phosphorus per gram of nitrogen. The part of the nitrogen balance caused by lymphoid tissue changes in the patient with chronic lymphatic leukemia was calculated by the formula $x = \frac{3N - 15P}{2}$ where x is the muscle nitrogen, N is the total nitrogen balance, and P is the phosphorus balance corrected for changes caused by movements of calcium. This formula is derived from the findings that the nitrogen:phosphorus ratio of lymphoid tissue is 5:1 and of muscle 15:1.[14]

Patient M. D. was a 56-year-old white woman with widely disseminated osteoblastic metastases from mammary carcinoma. The disease was confined to bones and soft tissue and caused little disability. The patient had been receiving 50 mg. of cortisone daily for 1 year and entered the hospital 1 month before the onset of the study. She was malnourished because of economic circumstances.

Patient A. C. was a 64-year-old white woman with mammary carcinoma metastatic to bone. Total hypophysectomy had been performed 3 months before the onset of this study. Maintenance therapy was cortisone, 12.5 mg. every 8 hours, and triiodothyronine, 25 mcg. every 8 hours. She had edema of both lower extremities secondary to thrombophlebitis.

Patient S. Y. was a 52-year-old white man with chronic lymphatic leukemia which was progressive and symptomatic. Previous therapy had included x-ray and triethylene melamine. He had many easily palpable and measurable lymph nodes, as well as hepatosplenomegaly and moderate edema of the lower extremities, presumably secondary to lymphatic obstruction.

RESULTS

From the derived data presented in Tables I and II, the effects of a daily dose of 100 mg. and 1,000 mg. of prednisone may be compared. Patient A. C. developed a total negative nitrogen balance of 21.2 Gm. in the 6 day period on the lower dose of prednisone and a total negative nitrogen balance of 19.9 Gm. when she received 1,000 mg. a day of this compound over another 6 day period. As the base lines of the control periods which preceded the test doses of prednisone were comparable, it is evident that the higher dose of prednisone had no greater catabolic effect. This patient who had undergone total hypophysectomy could achieve a mildly anabolic state when maintained with triiodothyronine and prednisone alone (Fig. 1).

In the second patient, M. D., 100 mg. of prednisone daily resulted in a negative nitrogen balance of 3.0 Gm., whereas 1,000 mg. of prednisone daily

‡Phosphorus not moving with calcium or nitrogen.
§Average daily value.

led to a negative nitrogen balance of 12.7 Gm. As the nitrogen balances during the preceding control periods were different, it is difficult to quantitatively compare these two dosage regimens. A marked positive nitrogen balance developed 3 days after the change of the prednisone dosage from 100 mg. per day to the control level of 15 mg. per day, and after 12 days on the latter dosage, nitrogen equilibrium had not yet been reached.

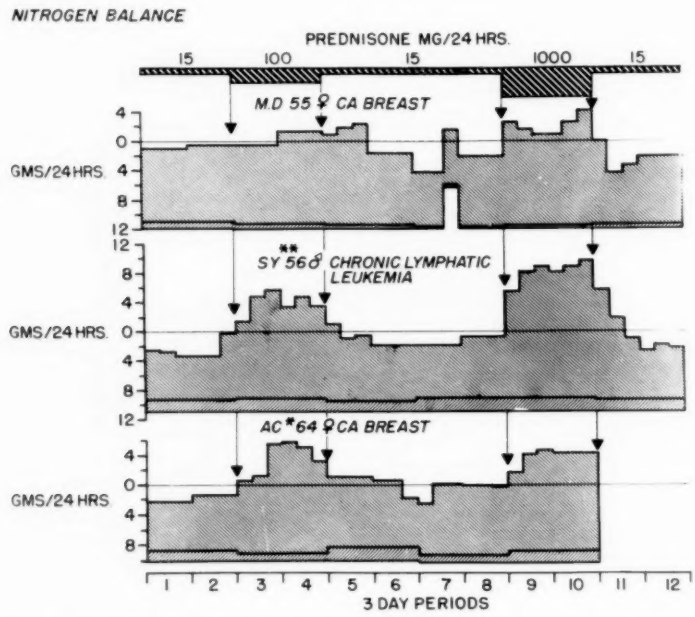

Fig. 1.—Effects of 100 mg. per day and 1,000 mg. per day of prednisone on nitrogen balance. The dietary intake is plotted down from the zero line; the fecal and urinary outputs are plotted on the intake line from below upward, starting with the fecal portion. *Cortisone acetate, 37.5 mg. per day, instead of prednisone, 15 mg. per day, for first 6 days. **Cortisone acetate, 50 mg. per day, instead of prednisone, 15 mg. per day, for first 6 days.

To correct for the differing base lines in this study, the nitrogen balance for the appropriate control period was subtracted from the balance during the test period. On the basis of these calculations, there was a total negative nitrogen balance of 7.5 Gm. when 100 mg. per day of prednisone was administered, whereas 1,000 mg. per day produced a total negative nitrogen balance of 26 Gm., a three-and-one-half-fold increase in nitrogen loss for a tenfold change in dosage of the drug.

When S. Y., the patient with chronic lymphatic leukemia, received 100 mg. of prednisone daily, the total negative nitrogen balance for the 6 days was 23.1 Gm. One thousand milligrams of prednisone per day resulted in a total negative nitrogen balance of 48.1 Gm.

Since there was measurable decrease in the adenopathy during these periods, the nitrogen balance was partitioned between lymphoid tissue and muscle (Table II). As there are data suggesting that large doses of cortisone can initially specifically affect the phosphorus balance independently of nitrogen

and calcium balance,[15] only the balance data of the last 3 days of each test period have been used. The lower dose of prednisone caused the catabolism of 1.8 Gm. of nitrogen daily derived from muscle and 2.0 Gm. of nitrogen daily derived from lymphoid tissue. At the higher dose of prednisone, the nitrogen from muscle was 5.8 Gm. daily, and from lymphoid tissue 3.0 Gm. daily.

This patient was in positive nitrogen balance before the study with the lower dosage of prednisone, and almost in nitrogen balance before the higher

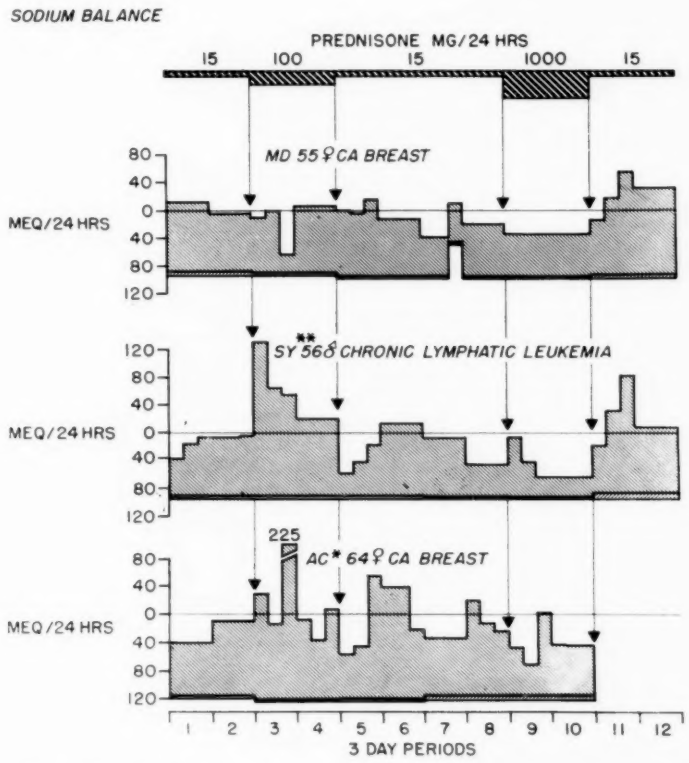

Fig. 2.—Effects of 100 mg. per day and 1,000 mg. per day of prednisone on sodium balance. *Cortisone acetate, 37.5 mg. per day, instead of prednisone, 15 mg. per day, for first 6 days. **Cortisone acetate, 50 mg. per day, instead of prednisone, 15 mg. per day, for first 6 days.

TABLE II. PARTITION OF AVERAGE DAILY NITROGEN BALANCE INTO MUSCLE-NITROGEN AND LYMPHOID TUMOR NITROGEN

PATIENT S. Y.	(1) FIRST CONTROL PERIOD: CORTISONE (50 MG./DAY)	(2) PREDNISONE (100 MG./DAY)	(3) (2) − (1)	(4) SECOND CONTROL PERIOD: PREDNISONE (15 MG./DAY)	(5) PREDNISONE (1,000 MG./DAY)	(6) (5) − (4)
Muscle N (Gm.)	+2.8	−1.8	−4.6	+1.1	−5.8	−6.9
Tumor N (Gm.)	−0.2	−2.0	−1.8	+0.5	−3.0	−3.5

Figures in Columns 3 and 6 represent changes in nitrogen balance caused by 100 mg. per day and 1,000 mg. per day of prednisone.

dosage was given. In order to correct for these differing base lines, the same calculation of the change of balance was applied as for Patient M. D.; the results are shown in Columns 3 and 6, Table II. This reveals that 1,000 mg. of prednisone caused only a moderately greater catabolic effect on muscle than did 100 mg., but that the breakdown of lymphoid tissue was doubled.

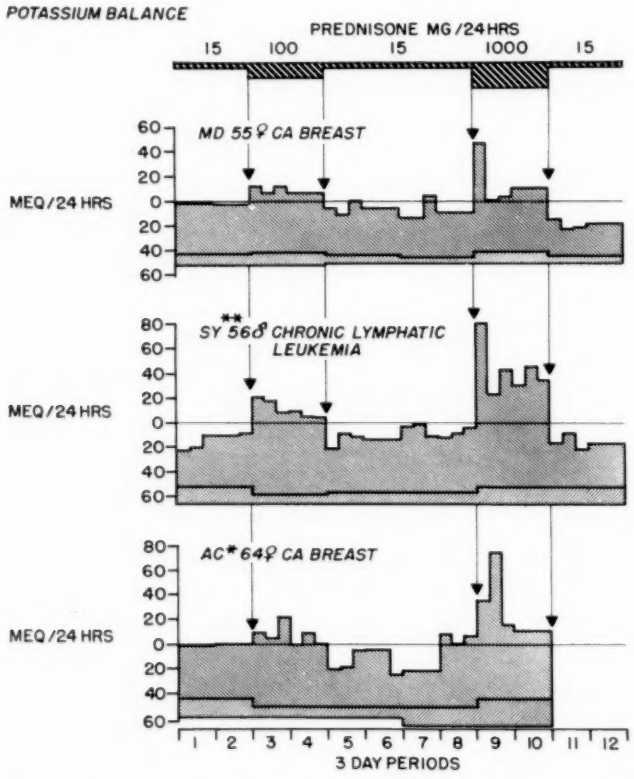

Fig. 3.—Effects of 100 mg. per day and 1,000 mg. per day of prednisone on potassium balance. *Cortisone acetate, 37.5 mg. per day, instead of prednisone, 15 mg. per day, for first 6 days. **Cortisone acetate, 50 mg. per day, instead of prednisone, 15 mg. per day, for first 6 days.

With respect to calcium balance, the minimal effects of massive dosages of prednisone are apparent. Patient M. D., who had osteoblastic bone disease, remained in positive balance throughout. The calcium balances in the fifth and sixth periods of S. Y.'s study suggest that a large part of the stool calcium of the fifth period was not passed until the sixth study period.

A comparison of the effects of the two dosages of prednisone on sodium balance is shown in Table I and Fig. 2. A retention of 228, 210, and 321 mEq. of sodium occurred in patients A. C., M. D., and S. Y., respectively, during the 6 day period on 1,000 mg. of prednisone daily. At the lower dosage of prednisone, Patients A. C. and S. Y. exhibited a negative sodium balance of 231 and 208 mEq., respectively, for the total 6 day period. These sodium losses were associated with the loss of leg edema which followed the shrinkage of inguinal

lymph nodes in Patient S. Y., and improvement of the phlebitic process in Patient A. C. On this same dosage, Patient M. D. exhibited an insignificant change in the sodium balance. Thus, at the lower dosage, no sodium retention occurred and 2 of the patients underwent natriuresis as the local factors improved. Even at the higher level the sodium retention was not profound, averaging 42 mEq. daily with an average daily sodium intake of 100 mEq. Serum electrolyte concentrations remained unchanged throughout the entire study for all patients with the one exception of a serum potassium of 3 mEq. per liter found in A. C. after the completion of the period on a higher dosage of prednisone.

Potassium balances were in the same direction as nitrogen balances. Calculations of theoretic potassium balances were not made because of the simultaneous alterations in sodium balance.

Absorption of the administered steroid was adequate as judged by the 24 hour urinary excretion of 17-hydroxysteroids.

The duration of effect of the different dosages of prednisone can best be evaluated from the balance charts. A carry-over effect on the nitrogen balance was observed to occur for 1 to 3 days after cessation of the test dose of the steroid (Fig. 1).

A marked potassium diuresis occurred during the first day or two on 1,000 mg. per day of prednisone in all 3 patients. A negative potassium balance was observed in each instance when 100 mg. of prednisone was given. The negative balance was twice as great after 1,000 mg. of prednisone was administered daily (Fig. 3). The early effect of administration of hydrocortisone-like steroids has been previously described, and its mechanisms of production have been discussed.[16, 17]

DISCUSSION

These studies demonstrate that although 1,000 mg. per day of prednisone may produce a catabolic effect of greater magnitude than would 100 mg. per day, this may not be marked, and the increase in catabolic effect, where observed, bears no relation to the dosage of this steroid. This may be the result of several factors.

A gradually diminishing effect with increasing doses of a hormone is commonly noted in biologic systems, the effect being proportional to the logarithm of the dose of the agent assayed. A maximum action is ultimately reached beyond which further increases in dosage produce no additional effect. The influence of the previous catabolic episode may not have been dissipated. However, there must be a maximum rate of muscle catabolism resulting from the administration of prednisone, and when this stage is reached, further increase in dosage would not result in further catabolic effect. Neither these data nor this hypothesis can explain the occasional requirements for amounts of corticosteroid much in excess of therapeutic dosages already considered large.

The catabolic effect on tumor tissue has been estimated in one patient, S. Y., from the results of the balance of nitrogen and phosphorus. The massive doses of prednisone may have effected a greater breakdown of tumor tissue than was obtained with the usual high dosage.

The changes in sodium balance produced by 100 mg. per day of prednisone were questionable in 2 patients, S. Y. and A. C., in whom changes in leg edema, secondary to circulatory impairment, may have greatly influenced this balance. In the third patient, M. D., who did not have such complicating factors, no significant change in sodium balance was observed when 100 mg. of prednisone was administered per day. When 1,000 mg. per day was given, on the other hand, all 3 patients exhibited sodium retention of moderate magnitude. Thus prednisone, like cortisone, had a sodium retaining effect. This was apparent only with massive doses.

No undesirable clinical effects occurred as a result of administration of either 100 or 1,000 mg. of prednisone daily. The abrupt decreases in dosage after each experimental period were well-tolerated.

SUMMARY

1. A comparison of the effects of prednisone in dosages of 100 mg. and 1,000 mg. daily was obtained by metabolic balance techniques in 3 patients. In one patient with chronic lymphatic leukemia, the effects of these steroids on the metabolism of tumor tissue were estimated.

2. In one patient, the catabolic effect of 1,000 mg. of prednisone was no greater than 100 mg. In 2 patients, the increment of catabolism which resulted from a dosage of 1,000 mg. of prednisone was not linearly proportional to the increase in dosage.

3. Similarly, when the effect on the lymphoid tumor tissue of one patient was calculated, it was found that a twofold increment in tumor tissue nitrogen loss occurred with a tenfold increase in the daily dosage of prednisone over 100 mg. per day.

4. Prednisone had an equivocal effect on the sodium balance when given at a dosage of 100 mg. per day. Moderate sodium retention occurred in all 3 patients, however, when 1,000 mg. per day was given.

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THE ANEMIA OF CIRRHOSIS

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The anemia of Laennec's cirrhosis was studied in 24 patients, with specific evaluation of the roles of hemolysis, hypervolemia, and chronic bleeding. Standards for the interpretation of the results were obtained by comparable determinations in suitable control subjects. The red cell volume and total blood volume were estimated from the dilution of transfused Cr^{51}-tagged red blood cells. Ten of 24 patients had a red cell mass below the normal range. Plasma volume, estimated by subtracting the red cell mass from the total blood volume, was increased in 15 of the 24 patients with cirrhosis. The red blood cell survival, determined with autogenous Cr^{51}-tagged red cells, indicated that 9 of the patients with cirrhosis had normal and 15 had decreased survival times. Analysis for radioactivity of the feces after the administration of Cr^{51}-tagged red cells indicated loss of blood into the gastrointestinal tract in 7 of 10 patients with cirrhosis studied in this manner. Thrombocytopenia, which was present in 12 of the 24 patients with cirrhosis, appeared to be associated with intermittent, unsuspected bleeding from the gastrointestinal tract.—Ed.

THE ANEMIA which occurs in Laennec's cirrhosis has been the subject of numerous investigations, and knowledge of its pathogenesis has evolved rapidly during the past two decades. As a result, several theories have been discarded, whereas others, such as lack of or failure to properly utilize vitamin B^{12}, have become debatable.[1-4] Hypervolemia of the plasma, formerly stressed as a paramount factor in the anemia,[5-7] has been overshadowed by the more recent observations of a hemolytic process in this disease.[9-12]

Little consideration has been given to chronic intermittent blood loss via the gastrointestinal tract as a contributing factor in the development of the anemia. In previous studies this complication was eliminated by selecting patients clinically free of hemorrhage or patients whose stools showed no occult blood. The difficulty in clinically assessing small amounts of blood lost intermittently from the gastrointestinal tract is obvious. Moreover, chemical determinations of blood in the stool depend on adequate sampling,[13, 14] which is particularly difficult when blood loss is intermittent.

Our purpose was to assess the incidence of anemia in cirrhosis and to investigate the roles of hemolysis, hypervolemia, and chronic bleeding in its pathogenesis.

METHODS AND MATERIALS

Twenty-four patients, 20 men and 4 women, with Laennec's cirrhosis were studied. Diagnosis was confirmed in each case by liver biopsy. All patients had experienced at least

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one episode of gastrointestinal hemorrhage, and 12 were hospitalized because of bleeding. Esophageal varices were demonstrated in all 24 patients by esophagoscopy. The subjects ranged in age from 29 to 69 years (average 41 years). Patients entered the study after they were clinically stabilized. They were ambulatory and without ascites. None had received a transfusion or shown evidence of hemorrhage for at least 3 months.

Twenty normal subjects and 17 patients with untreated polycythemia were used as controls for blood volume determinations. Previous studies have revealed that polycythemia vera is characterized by an increase of the red cell mass at the expense of the plasma volume. The availability of a group of patients with this disease encouraged us to utilize them as additional controls in the plasma volume studies.

Ten hospitalized cirrhotic patients and 5 control subjects were observed for intestinal blood loss after transfusion of autogenous red blood cells labeled with Cr^{51}.

Four normal subjects were subjected to repeated venesection during a red blood cell survival study.

Weekly hemograms consisting of a red blood cell count, hemoglobin, hematocrit, reticulocyte count, and platelet count were done by standard techniques.[15] Bone marrow was aspirated from the sternum.

The red blood cell volume and total blood volume were estimated from the dilution of transfused Cr^{51}-tagged red cells.[16] Plasma volume was estimated by subtracting the red cell mass from the total blood volume. Autogenous Cr^{51}-tagged erythrocytes were used to determine red blood cell survival.[17] Five milliliter blood samples were counted simultaneously with the initial postinjection sample: residual radioactivity was expressed as percentage of the initial sample.[18]

The biologic half-life (T/2) of Cr^{51}-tagged red cells varies from 25 to 40 days.[19-21] The effect of chronic blood loss on the survival of labeled cells was determined by repeatedly bleeding 4 normal subjects after transfusion of Cr^{51}-tagged autogenous red cells. Aliquots of 15 and 50 ml. of blood were removed, respectively, from each of 2 young men between the tenth and fifteenth days of the Cr^{51} red cell survival study.

Fecal analysis for radioactivity after the administration of Cr^{51}-tagged red cells afforded a quantitative measurement of gastrointestinal blood loss from the gastrointestinal tract in 10 cirrhotic patients. After initiation of a Cr^{51} red cell survival study, at least five 24 hour stool specimens were collected from each patient and analyzed for radioactivity by use of the method described by Bannerman.[13] Blood lost in the 24 hour fecal specimens was computed from the radioactivity, compared with that of the blood, and multiplied by the weight of the 24 hour stool specimens. After the 24 hour stool specimen was weighed, but before emulsification, a portion of the stool was selected at random and forwarded to the hospital laboratory for an independent guaiac determination for occult blood.

Total protein and plasma albumin levels were measured on the day blood volume determinations were done. The plasma volume and albumin values were used to compute the plasma albumin per kilogram of body weight as noted:

$$\text{Plasma volume}\left(\frac{\text{milliliter}}{\text{kilogram body weight}} \times \text{plasma albumin}\ \frac{\text{gram}}{\text{milliliter}}\right) = \text{gram plasma albumin per kilogram of body weight.}$$

RESULTS

Table I contains the results of the red cell mass (R.C.M.) and plasma volume (P.V.) determinations. The mean value of the R.C.M. was 25.4 ± 5 ml. per kilogram of body weight in the cirrhotic patients, 29.8 ± 3.0 ml. per kilogram in the normal subjects, and 47.9 ± 6 ml. per kilogram in the polycythemic patients. There was no overlap in the mean values or the 95 per cent confidence limits in any of the 3 groups, which indicates a significant difference in the mean values.

TABLE I. COMPARISON BETWEEN THE RED CELL MASS, PLASMA VOLUME, AND TOTAL BLOOD VOLUME IN PATIENTS WITH CIRRHOSIS WITH POLYCYTHEMIA VERA, AND NORMAL INDIVIDUALS

	CIRRHOSIS				NORMAL				POLYCYTHEMIA VERA		
CASE	RED CELL MASS (ML./KG.)	PLASMA VOLUME (ML./KG.)	TOTAL BLOOD VOLUME (ML./KG.)	T/2 Cr^{51} (DAYS)	RED CELL MASS (ML./KG.)	PLASMA VOLUME (ML./KG.)	TOTAL BLOOD VOLUME (ML./KG.)	T/2 Cr^{51} DAYS)	RED CELL MASS (ML./KG.)	PLASMA VOLUME (ML./KG.)	TOTAL BLOOD VOLUME (ML./KG.)
1	32.0	44.3	76.3	25.0	27.9	36.3	64.2	29	46.2	44.3	90.5
2	24.5	32.5	56.9	29.0	27.1	32.5	59.6	25	62.8	30.9	93.7
3	25.5	39.8	65.3	28.0							
4	32.5	60.3	92.7	24.0	31.6	41.6	73.2	33	48.3	42.9	91.2
5	30.4	53.1	83.5	25.0	35.0	46.2	81.2	28	44.5	37.0	81.5
6	26.5	54.2	80.7	24.0	33.4	43.0	76.4	30	49.5	42.7	92.2
7	27.7	50.3	78.1	24.0	33.2	42.9	76.1	25			
8	30.9	62.7	93.6	19.0	31.3	42.8	74.1	27	58.3	30.7	89.0
9	24.9	66.2	91.1	21.0	31.4	40.4	71.8	24	43.6	40.3	83.9
10	25.7	50.5	76.2	21.0	26.2	44.5	70.7	29	39.7	31.8	71.5
11	25.7	60.2	85.9	22.0	33.1	39.6	72.7	30	41.9	40.2	82.1
12	38.9	60.7	99.7	22.0	33.7	41.9	75.6	26	47.7	48.5	96.2
13	25.5	56.8	82.4	13.0	28.2	39.2	67.4	27	45.7	38.0	83.7
14	25.8	55.0	80.8	23.0	25.6	41.9	67.5	27			
15	20.5	41.2	61.7	23.0	24.5	45.5	70.0	27	42.4	35.2	77.6
16	20.9	41.2	62.2	23.0	29.9	42.3	72.2	30	51.9	43.2	95.1
17	24.2	42.5	56.4	25.0	29.5	44.2	73.7	24			
18	21.7	45.0	66.7	16.0							
19	17.2	45.8	63.0	22.0							
20	23.7	36.9	60.6	22.0							
21	20.5	66.3	86.8	12.0	28.7	33.3	62.0	27	53.9	39.1	93.0
22	20.9	50.8	71.7	24.0	26.2	35.7	61.9	26	40.9	49.0	89.9
23	20.5	58.4	78.9	16.0	29.2	46.8	76.0	26	46.3	41.1	87.4
24	22.7	55.5	78.2	16.0	30.5	38.7	69.2	24	51.0	45.8	96.8
SY	609.3	1230.2	1839.3	519.0	596.0	819.3	1415.3	572	814.6	680.7	1495.3
S.D.	±4.86	±7.25	±11.85		±3.01	±4.04	±5.61	±2.4	±6.21	±5.62	±7.00
Mean	25.38	51.26	76.65	21.6	29.81	40.96	70.77	27.2	47.91	40.04	87.95
95% Confidence limits	23.3–27.4	48.2–54.3	71.6–81.7		28.4–31.2	39.1–42.8	68.1–73.4		43.7–51.1	37.1–42.9	84.4–91.5

Fourteen (58 per cent) of the cirrhotic patients had a normal red cell mass, if the lowest figure (24.5 ml. per kilogram) in the control group was considered the limit of normal. Ten patients (42 per cent) had a R.C.M. below this value. The hematocrits obtained at the time the R.C.M. was determined are shown in Table II. There was poor correlation between these 2 values. Seventeen (70 per cent) of the patients with normal red cell volumes had normal red cell survival; the other 7 had abnormal erythrocyte survival times. Two patients with decreased red cell masses had normal red cell survival times (Cases 17 and 22).

Plasma Volume.—The mean plasma volume for the group of patients with cirrhosis was 51.2 ± 7.3 ml. per kilogram of body weight. This was significantly greater than the mean values for the plasma volume in the control subjects and polycythemic patients. A substantial overlap existed between the mean plasma volume values and the 95 per cent confidence limits of the latter groups. There was no overlap between the mean plasma volume of the cirrhotic patients, and those of either of the other 2 groups.

An increased plasma volume was present in 15 (63 per cent) of the cirrhotic patients. Of the 24 cirrhotic patients 3 had normal R.C.M. and P.V., 11 had normal R.C.M. and increased P.V., 6 had decreased R.C.M. and normal P.V., and 4 had decreased R.C.M. and increased P.V. (Table II).

Red Cell Survival.—The red cell half-life determined with autogenous Cr^{51}-tagged red cells ranged from 12 to 28 days in the cirrhotic patients and from 24 to 33 days in the control subjects. The mean half-life of the erythrocytes in the control subjects was 27.2 ± 2.4 days. This agrees with determinations by Mollison and Veall,[20] 25 ± 1.5 days; Donohue and co-workers,[17] 26 ± 2 days; and Crawford and de Gruchy,[16] 26 ± 3 days. Considerable variation in red cell survival was found in the patients with cirrhosis. Nine patients had normal and 15 had decreased survival times. The red cell half-life was 23 days in three patients, 22 days in four, 21 days in two, 19 days in one, and 16 days or less in five patients.

Blood Loss as Determined by Radioactivity in the 24 Hour Stool Specimens.—The 5 normal subjects studied in this manner showed radioactivity in their stools consistent with a loss of 0.75 to 1.45 ml. (mean 1.3 ml.) of whole blood per day, which agrees with the observations of Ebaugh and associates.[14]

The results obtained by examination of the 24 hour stool specimens of the 10 patients with cirrhosis are shown in Table III. Three patients developed evidence of hemorrhage at the time the survival studies were in progress (Cases 8, 14, and 24). One of these (Case 14) was ambulatory, and his episode of intestinal blood loss was not suspected by the attending physician. Intermittent fecal blood loss was found in 5 patients (Cases 4, 9, 12, 14, and 21). All but one had previously undergone portacaval shunt procedures. Three patients showed no appreciable loss of blood in stools (Cases 1, 3, and 20) and 2 of these had previously undergone shunt procedures. The quantity of blood lost via the stool was not reflected by the guaiac test for occult blood.

TABLE II. LABORATORY DATA O

CASE	RED CELL MASS (ML./KG.)	PLASMA VOLUME (ML./KG.)	Cr^{51} (DAYS) –50% SURVIVAL	R.B.C. (10^6/C. MM.)	HCT (%)	H.B. (GM./100 ML.)	RETICULOCYTES (%)	PLATELETS ($\times 10^3$ PE C. MM.)
	Normal	Normal						
1	32.0	44.3	25	4.0	33	10.7	1.1	67
2	24.5	32.5	29	5.1	47	16.1	0.7	180
3	25.5	39.8	28	5.5	49	14.9	2.0	80
	Normal	Increased						
4	32.5 (1957)	60.3	24	4.2	39	11.2	1.8	45
	24.9 (1958)	58.3	18	3.6	33	9.1	3.8	400
5	30.4	53.1	25	4.6	43	14.0	1.2	235
6	26.5 (1957)	54.2	24	4.1	39	11.8	1.6	120
	28.0 (1958)	57.4	25	4.3	39	12.1	4.0	249
7	27.7 (1956)	50.3	24	3.4	34	11.7	3.3	94
	34.2 (1957)	76.2		4.0	36	11.8	4.7	78
8	30.9 (1956)	62.7	19	4.5	42	14.0	0.8	46
	25.8 (1957)	55.1	22	4.2	41	12.3	2.7	75
9	24.9	66.2	21	3.9	34	10.9	1.4	98
10	25.7 (1956)	60.2	21	3.3	40	12.2	3.1	81
	24.6 (1957)	40.2	24	4.6	42	14.8	2.3	110
11	25.7	60.2	22	3.7	34	10.3	1.6	248
12	38.9	60.7	22	4.6	41	12.8	3.1	32
13	25.5	56.8	13	3.9	34	11.4	1.7	150
14	25.8 (1957)	55.0	23	3.5	35	12.4	1.9	40
	26.2 (1958)	58.5	23	3.6	34	10.7	2.8	60
	17.3 (1958)	61.9	8	2.5	24	8.7	3.7	75
	Decreased	Normal						
15	20.5	41.2	23	4.1	37	13.4	3.2	150
16	20.9	41.2	23	4.8	37	12.4	1.9	58
17	24.2	42.5	25	4.0	37	12.7	1.7	280
18	21.7	45.0	16	4.0	36	12.6	0.8	90
19	17.2	45.8	22	2.9	30	10.8	2.3	200
20	23.7	36.9	22	4.1	43	13.7	1.8	265
	Decreased	Increased						
21	14.0 (1956)	55.2	12	2.9	29	9.0	2.9	123
	20.5 (1958)	66.3	14	2.7	27	8.1	5.4	169
22	20.9	50.8	24	3.3	34	9.3	6.4	292
23	20.5 (1957)	58.4	16	2.3	26	7.8	8.8	40
	28.8 (1958)	86.3	24	4.9	41	13.0	4.0	466
24	22.7 (1957)	55.5	16	2.5	26	7.6	3.2	55
	24.8 (1957)	54.3	25	3.7	32	12.0	1.4	240

MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean
*E.H. = erythroid hyperplasia.

The Effect of Blood Loss on the Red Cell Survival Curve.—Loss of radioactivity in the stools may have contributed to the abnormal red cell survival noted in Cases 4, 8, 9, 14, and 24 (Table III). The apparent reduction in the half-life of the Cr^{51}-tagged red cells of 3 patients was converted to normal (Cases 4, 9, and 14) when corrected for the average radioactivity lost per day in the stools. This procedure lacked accuracy (and was not valid) because only five 24 hour stool specimens were collected in these cases. Moreover, inspection of the red cell survival curves of the 4 volunteers who were bled 15 and 50 ml. daily for 6 days (Fig. 1) revealed no appreciable alteration in the shape or length of the survival curves. This was notable because 300 ml. of blood had been withdrawn from 2 of the subjects over a 6 day period.

24 Patients With Cirrhosis

MCV c. u.	MCH u. u. g.	MCHC (%)	Indirect bilirubin (mgm. %)	Prothrombin time (%)	Hepatomegaly	Splenomegaly	Bone marrow	Total protein albumin (Gm./100 ml.)	Albumin (Gm./Kg.)
83	27	32	0.8	50	X	X	E. H.*	8.3/3.4	1.5
92	31	34	0.6	95	–	–		8.3/3.9	1.3
90	27	30	0.4	85	X	X	Normal	6.0/3.0	1.2
93	26	31	0.9	45	X	–	E. H.		
91	25	27	0.7	48	X			6.3/2.1	1.2
93	30	32	0.8	75	X	–	Normal	7.5/4.0	2.1
95	29	30	1.3	60	X	X	E. H.	7.0/2.5	1.4
91	28	31	1.0	45	X	X			
100	34	34	3.2	38	–	X	E. H.	8.5/2.9	1.5
90	30	32	2.9	30	–	X			
93	31	33	0.4	38	X	X	E. H.		
97	30	30	3.1	28	X	X		7.6/2.2	1.2
87	30	32	2.8	50	X	X	E. H.		
121	37	31	0.7	90	X	X	E. H.	7.5/3.4	2.0
91	32	34	0.4	92	X	X		8.1/3.1	1.3
92	28	30	0.5	90	X	–	Normal	7.4/3.1	1.9
89	28	31	1.0	68	X	X	E. H.	6.4/2.4	1.5
87	30	33	2.3	30	X	X	E. H.	6.6/2.2	1.3
100	35	35	1.8	60	X	X	E. H.	6.0/2.5	1.4
94	29	31	1.0	45				6.4/2.4	1.4
96	34	36	0.5	30				6.7/3.3	2.0
90	32	36	1.0	50	X	X	E. H.	8.0/3.4	1.4
75	26	33	0.4	85	X	–	E. H.	7.7/3.4	1.4
92	32	34	0.6	80	X	–	Normal		
90	32	35	0.5	35	X	X	E. H.	7.2/3.0	1.3
103	37	36	0.6	90	X	X	E. H.	6.4/2.2	1.0
104	33	32	0.9	75	X	–	–		
103	32	31	3.2	35	X	X	E. H.		
100	30	30	2.7	52	X	–	E. H.	6.3/1.7	1.1
103	28	27	0.8	35	X	–		7.2/3.1	1.6
113	24	32	4.2	45	X	X	E. H.	5.9/2.7	1.5
83	27	31	0.8	67	X	–		6.7/3.0	2.5
102	30	30	0.8	90	X	X	E. H.		
87	32	37	0.5	95	X	X			

* normocorpuscular hemoglobin concentration.

Thrombocytopenia.—Fifty per cent of the patients had a platelet count below 100,000 per c.mm. Thrombocytopenia was present in 9 of the 10 patients who had Cr^{51} stool determinations. Four patients had an increase in platelet count after a portacaval shunt, whereas 4 continued to have thrombocytopenia after similar operative procedures (Table III). This same procedure alleviated pancytopenia in Case 25, and a postoperative red cell survival was normal. Pancytopenia persisted in Case 4 after the portacaval procedure, and 8 months later a splenectomy was required. Splenectomy was followed by a return of the red cell half-life to normal in Cases 12 and 23. These findings appear to support Jandl's concept of splenic sequestration as a cause of the hemolytic process in cirrhosis.[12]

TABLE III. THE RELATION OF THE Cr^{51} 50 PER CENT RED CELL SURVIVAL TIME TO GASTROINTESTINAL BLOOD LOSS AND THROMBOCYTOPENIA IN CIRRHOSIS

PATIENT NO.	T/2 Cr^{51} R.B.C. (DAYS) PREOPERATIVE	T/2 Cr^{51} R.B.C. (DAYS) POSTOPERATIVE	PLATELETS ($\times 10^3$ PER C.MM.) PREOPERATIVE	PLATELETS ($\times 10^3$ PER C.MM.) POSTOPERATIVE	24 HR. FECAL (Cr^{51}) BLOOD LOSS PREOPERATIVE DAY OF SURVIVAL	ML.	GUAIAC	24 HR. FECAL (Cr^{51}) BLOOD LOSS POSTOPERATIVE DAY OF SURVIVAL	ML.	GUAIAC	OPERATION
1		25	43-67	154-210				2	1.2	0	Portacaval shunt
								4	2.1	++	
								6	1.3	0	
								8	1.4	0	
								10	1.9	+	
									7.9		
3	28	25	50-75	180-260				2	0.7	0	Portacaval shunt
								3	0.8	+	
								4	2.1	0	
								5	0.8	0	
								10	1.2	0	
									5.6		
4	24	18 25*	35-45	50-60				1	3.6	++	Portacaval shunt
								2	4.7	0	
								3	2.1	0	
								4	2.1	0	
								10	2.5	0	
									15.0		
8	19	22	55-65	60-70				1	2.0	0	Portacaval shunt
								2	1.4	0	
								3	1.6	0	
								4	4.9	+++	
								6	69.7	++++	
								7	195.2	++++	
								8	133.2	++++	
								16	20.3	++++	
									428.3		
9		21 27*	75-90	80-120				2	54.0	+++	Portacaval shunt
								3	11.4	+	
								6	6.3	+	
								9	14.8	+	
								10	6.0	0	
									92.5		
12	23	26	25-38	275-450	3	0.9	0				Splenectomy
					5	0.5	0				
					7	4.6	0				
					9	4.6	0				
					11	3.2	++				
						15.8					
14	23 (1951)	8	45-55	65-75	2	11.1	+++	2	258.0	++++	Portacaval shunt
					3	10.9	++	3	1.2	0	
					4	3.4	+	4	2.8	+	
	23 (1958)				5	24.8	+	5	21.3	+	
					6	4.0	0	10	19.7	+++	
	26*				14	0.5	0		305.0		
						54.7					
20	22		250-325	300-420	2	1.0	0				None
					3	0.9	0				
					4	1.3	+				
			250-420		5	1.2	0				
					8	0.5	0				
						4.9					

*Fifty per cent survival time corrected for estimated fecal loss.

TABLE III. (CONT'D)

PATIENT NO.	T/2 Cr^{51} R.B.C. (DAYS) PREOPERATIVE	T/2 Cr^{51} R.B.C. (DAYS) POSTOPERATIVE	PLATELETS ($\times 10^3$ PER C.MM.) PREOPERATIVE	PLATELETS ($\times 10^3$ PER C.MM.) POSTOPERATIVE	24 HR. FECAL (Cr^{51}) BLOOD LOSS PREOPERATIVE DAY OF SURVIVAL	ML.	GUAIAC	POSTOPERATIVE DAY OF SURVIVAL	ML.	GUAIAC	OPERATION
21	12	14	50-90	110-158				2	21.3	+	Portacaval shunt
								4	7.9	++	
								5	24.6	+++	
								7	1.2	0	
								8	1.9	0	
									56.9		
23	16	24	35-40	260-315							Splenectomy
24	16	25	30-50	140-250	2	4.2	++	2	1.1	0	Portacaval shunt
					3	3.1	+	3	1.0	+	
					4	10.5	++	4	0.8	0	
					5	20.2	++	5	0.5	0	
					7	290.0	++++	6	0.4	0	
						328.0			3.8		

The single patient without thrombocytopenia showed no loss of excess radioactivity in stools.

DISCUSSION

Anemia (R.C.M. 24.5 ml. per kilogram or less) was found in 42 per cent of the patients with cirrhosis. This incidence agrees with the observations of Cachera (40 per cent) and Eisenberg (24 per cent),[22, 23] but is far less than the incidence of anemia reported in previous studies.[24, 25] The latter reflects the unreliability of hematocrit and hemoglobin determinations as measures of anemia in cirrhosis. Seventy per cent of our cirrhotic patients would have been considered anemic, if a hematocrit of 40 per cent was the criterion for anemia. A relatively high hematocrit masks the severity of most chronic anemias.[26] However, in cirrhosis, the hypervolemia accentuates the decreased red cell volume, and may suggest anemia in the presence of a normal red cell mass.

The expanded plasma volume in cirrhosis has been attributed to both hypoalbuminemia and extensive portal-collateral circulation.[5] But the development of the latter in extrahepatic obstruction is not accompanied by hypervolemia,[27] and the hypoalbuminemia of cirrhosis may be a relative deficiency. Half the body pool of albumin is intravascular and the turnover of plasma albumin is not altered in the disease.[28, 29] The total amount of circulating protein was less than normal (in terms of grams per kilogram of body weight) in only 2 patients (Table II). In the other patients when plasma volume had expanded, the mass of protein had not. The low concentration of plasma protein appeared to be a result of dilution. It is interesting to speculate that "ascites of the blood" may be the precursor of clinical ascites.

The red cell survival was decreased in the majority (62 per cent) of our cirrhotic patients. Repeated studies at different intervals with Cr^{51}-tagged red cells have shown that the severity of the hemolytic phenomenon in such patients

varies considerably from time to time. Hemolytic disease may disappear completely after portacaval operation in some cirrhotic patients, whereas only splenectomy eliminates it in others. Intermittent bleeding from the intestinal tract can magnify the effect of hemolysis. However, considerable blood loss is necessary before the survival cure of tagged red cells is altered. In 2 normal subjects a loss of 300 ml. of blood in 6 days during the Cr^{51} study did not appreciably affect the half-life of the red cells.

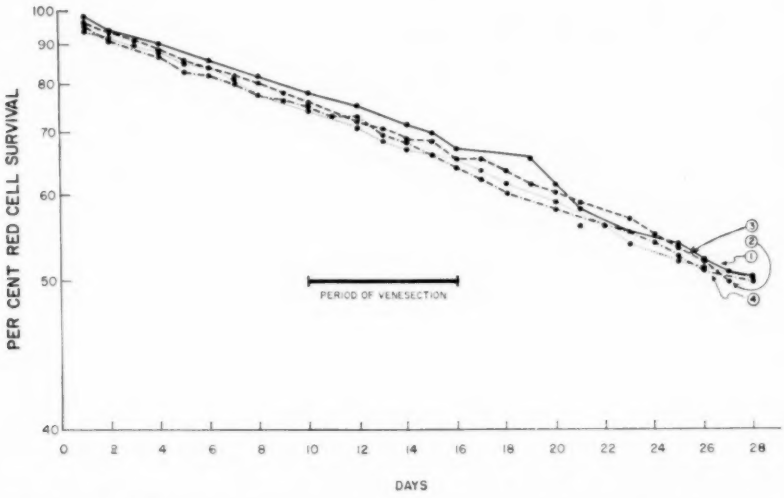

Fig. 1.—The effect of daily venisection on the curve of decay of autotransfused red cells labeled with Cr^{51}. Four normal subjects were so transfused, and for 10 days thereafter the slope of the curve was established. Then for 1 week they were bled each day, after which the slope of the curve was once again determined. Subjects 1 and 2 were bled 50 ml. per day; Subjects 3 and 4 were bled 15 ml. per day. It is apparent that this amount of blood loss is insufficient to alter the slope of the curve of decay of Cr^{51}-tagged red cells in normal subjects.

Demonstrable intestinal lesions may produce intermittent blood loss, and a duodenal ulcer caused this in 2 patients. In the remaining subjects with this type of blood loss, inspection of the esophageal varices, extensive radiologic examinations of the gastrointestinal tract, and the measurement of radioactivity in 12 hour gastric aspirations failed to reveal a localized source of hemorrhage. Thrombocytopenia was the common denominator in those with intermittent loss of blood in stools. This was not surprising, for thrombocytopenia is quite common in cirrhosis,[30-32] and is often associated with hemorrhagic diseases in these patients.[33] A tendency to mucosal bleeding and the fact that significant interstitial hemorrhage occurs long before clinical evidence of bleeding with thrombocytopenia makes the intestinal tract with its myriads of capillaries and extensive mucosal surface an ideal site for bleeding. Recovery from thrombocytopenia may follow portacaval operation, but is unpredictable. Only 4 of 8 patients with thrombocytopenia had a sustained rise in platelets after such operative procedures.

The anemia of cirrhosis is unusual. Hemolysis, hemorrhage, and chronic blood loss from thrombocytopenia may all contribute to it. However, even these

factors do not explain all the facets of the anemia. Some cirrhotic patients are unable to maintain a normal red cell volume, even though the hemolytic process which affects the red cells is no more severe than that of other cirrhotic patients with a normal red cell mass. This leads to another phase of the anemia, "a modified inhibition of bone marrow production." Normally, hemolytic disease is compensated by increased hemoglobin production. Under proper conditions hemoglobin production may increase sevenfold,[34] anemia developing only when the capacity for compensation is exceeded. In cirrhosis, this compensating mechanism is sometimes altered. The result is a limited increase in hemoglobin output, which is insufficient to compensate for the loss of red cells and maintain a normal circulating red cell volume. For example, in Case 20 there was a mean red cell life span of over 50 days.[17] A twofold increase in hemoglobin production should have been adequate to compensate for a hemolytic disease of this severity. Nonetheless the circulating red cell mass remained low.

Limitations of the bone marrow output may result from several deficiencies. One of these is lack of iron,[35] and the serum iron is often decreased in cirrhosis.[12] Indeed, superimposed iron deficiency may be associated with the macrocytosis so frequently encountered in the disease.[36]

CONCLUSION

The pathogenesis of the anemia of cirrhosis is complex. Blood loss, a hemolytic component, and partial inhibition of the bone marrow may singly or together contribute to the anemia. Hypervolemia accentuates both the incidence and severity of low values for hemoglobin and hematocrit in patients with this disease.

When thrombocytopenia complicates the disease there is often intermittent, unsuspected bleeding from the gastrointestinal tract.

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RATE OF DEVELOPMENT OF RESISTANCE IN STAPHYLOCOCCUS AUREUS TO ERYTHROMYCIN IN COMBINATION WITH OLEANDOMYCIN, VANCOMYCIN, OR DIHYDROSTREPTOMYCIN

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The rate of emergence of resistance to various antibiotics was measured for 35 strains of virulent staphylococci by three methods. Technical factors which influenced the apparent capacity of staphylococci to develop resistance to antibiotics included such variables as the concentration of the antibiotic, its activity, the medium employed, the size of the bacterial population, time of bacterial exposure to the antibiotic, bacterial sampling techniques, variation between bacterial strains, and variation in bacterial clones within a strain. The influence of these variables was apparent in studies in which single antibiotics as well as combinations of antibiotics were used. The combination of equal weights of erythromycin and oleandomycin delayed the emergence of resistance in most strains of staphylococci upon serial transfer. Vancomycin also retarded adaptation of staphylococci to erythromycin, as expected. Vancomycin also seemed to delay resistance to dihydrostreptomycin but it had no such effect on resistance to oleandomycin. Resistance to erythromycin plus dihydrostreptomycin usually developed at a slower rate than to either drug alone. Oleandomycin-dihydrostreptomycin mixtures delayed the emergence of resistance on serial transfer but the rate of adaptation was rapid after a few transfers. Preliminary studies did not show a close relation between the rate of mutation and the rate at which resistance emerged on serial transfers.—ED.

TODAY the majority of strains of staphylococci which cause infections in hospitals are resistant to high concentrations of penicillin, streptomycin, and the tetracyclines. It has been proved that resistance to antibiotics may arise either from selection of resistant genetic mutants, a biochemical adaptation in the bacteria, or contamination of an infected lesion with resistant bacteria. In many hospitals, infections with antibiotic-resistant staphylococci have become a frequent and serious problem.

Many students of microbial genetics argue that antibiotic resistance may be prevented or retarded by the administration of antibiotics in combinations. This seems to be a sound preventive measure but there is little evidence that the antibiotic resistance of staphylococci in man has been measurably decreased by these combinations.[1, 2]

The rate at which resistance to an antibiotic may emerge in bacteria is usually measured by exposure of those bacteria to increasing amounts of the antibiotic over a period of time. During the past 3 years we have conducted

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35 experiments of this type with 15 strains of hospital staphylococci using antibiotics singly or in paired combinations. Mutation rates for resistance to some antibiotics were also determined by the method of Luria and Delbrück.[8a]

MATERIALS AND METHODS

Cultures.—The strains of *Staphylococcus aureus* employed were selected from 30 strains of pathogenic staphylococci recovered in 1954 from the blood or exudates of infected patients.[6] The cultures were stored at 10° C. on brain heart infusion (B.H.I.) agar slants. They were transferred to fresh medium approximately every 2 months.

Antibiotics.—The antibiotic solutions were usually prepared by dissolving a soluble crystalline powder of known potency in sterile distilled water at 28° C. The following antibiotics were used: potassium penicillin G (Lederle), dihydrostreptomycin sulfate (Sharp & Dohme), erythromycin glucoheptonate, erythromycin, erythromycins A and B (Eli Lilly), oleandomycin phosphate (Pfizer), neomycin sulfate (Upjohn), and vancomycin hydrochloride (Eli Lilly). The concentrated stock solutions were stored at −10° C. for not more than 1 week.

Induction of Resistance in Broth Flasks (Method 1).—The method was modified from that of Spink and Ferris.[3c] A loopful of culture from a B.H.I. agar slant was transferred to 5 ml. of B.H.I. broth (Difco) and incubated 18 hours at 37° C. To a 50 ml. Erlenmeyer flask were added 1 ml. of broth culture, 48 ml. of B.H.I. broth, and 1 ml. of antibiotic solution. The final concentration of antibiotic was adjusted so as to be slightly below the minimal inhibitory concentration. After the contents were mixed, the flask was incubated at 37° C. until full growth occurred (24 to 72 hours). A loopful was then transferred to a B.H.I. agar slant and 1 ml. was inoculated into 5 ml. of B.H.I. broth. These subcultures were incubated 18 hours at 37° C. The agar slant was checked for purity by the colonial and microscopic morphogenesis and by coagulase production. The broth subculture was used for inoculation of a double concentration of antibiotic. Such experiments were carried on for 15 to 20 transfers or until the staphylococci ceased to grow vigorously. The apparent resistance of staphylococci growing in antibiotic broth was confirmed by the broth dilution method of Rammelkamp and Maxon[4] as modified by Waisbren and associates.[7]

Induction by Serial Broth Dilution (Method 2).—This means of inducing antibiotic resistance has been widely used and is adapted from that of Rammelkamp and Maxon.[4] The stock antibiotic solution was diluted so that the final concentration would differ by twofold steps from about 0.01 to 100 mcg. per milliliter. To each dilution (0.5 ml.) of antibiotic in a sterile Kahn tube was added 0.5 ml. of a 1:10 broth dilution of an 18 hour B.H.I. broth culture of staphylococci. After mixing, the tubes were incubated 48 hours at 37° C. A loopful from the tube which contained the highest concentration of antibiotic which permitted full growth was transferred to a slant of B.H.I. agar to check for contamination. The remainder of the contents of this tube was poured into 10 ml. of B.H.I. broth. After 18 hours at 37° C. the latter was used to inoculate a new series of antibiotic dilutions. This process was repeated until full resistance to 100 mcg. per milliliter of antibiotic was reached or until 10 transfers had been completed.

Induction by Serial Agar Plate Dilution (Method 3).—This method was slightly modified from the procedure of Murray and co-workers.[5] A loopful of the test culture was transferred from B.H.I. agar to broth and was incubated overnight. A loopful was also streaked on a blood agar plate. After 24 hours 6 individual colonies were picked and transferred to 6 tubes of B.H.I. broth. After 24 hours these broth cultures were used as bacterial inocula.

B.H.I. agar (9 ml.) was dispensed into 70 ml. Pyrex tubes and was sterilized by heat. After it was melted, the agar was kept at 55° C. in a water bath. One milliliter of antibiotic solution was added to each tube and mixed with agar. The contents of each tube was then poured into a Petri dish and allowed to cool for 3 hours at about 28° C. The bottom of each plate was marked off into 6 equal segments. Each segment was streaked

with a 24 hour broth culture of the test strain. The plates were incubated 48 hours at 37° C. The amount of growth was recorded on a scale of 1 to 4 plus, resistance being indicated by 1 plus or greater growth.

Further exposure to the antibiotic was carried out by selecting the plate which contained the highest concentration of antibiotic that allowed growth of typical large, opaque colonies of staphylococci. Growth from the plate was inoculated into B.H.I. broth and incubated 24 hours. This broth was then used to inoculate the second group of antibiotic plates. From 13 to 15 serial transfers were carried out, unless complete resistance to 100 mcg. per milliliter developed earlier.

Mutation Rate (Negative Tube Method).—A single colony was picked from an agar culture of Strain 14 and inoculated into 10 ml. B.H.I. broth. After incubation for 24 hours the culture was centrifuged at 2,100 r.p.m. for 25 minutes. The supernatant (0.25 ml.) was transferred to each of three 15 ml. tubes of broth. After incubation to full growth the cultures were mixed and centrifuged as before. The first tube was used to inoculate fresh broth tubes for future experiments. Supernatants (1 ml.) from Tubes 2 and 3 were added to 9 ml. broth, mixed, and diluted serially to 1:10,000. The final volume in the last tube was 20 ml. Exactly 0.25 ml. of each of the two 1:10,000 dilutions was delivered to each of four 20 ml. tubes and fifty 5 ml. tubes. These were incubated 48 hours or until bacterial colonies were fully grown.

Viable Count.—The volumes in each of the 2 series of 4 large tubes were brought to 10 ml. with broth, mixed, and carried through 4 serial 1:10 dilutions in broth. A separate pipette was used for each dilution. Two pour plates were prepared with 0.5 ml. of the last dilution and 1 with 0.5 ml. of the next lower dilution; 10 ml. melted B.H.I. agar cooled to 56° C. was added. The 12 plates were incubated 24 hours and the visible colonies were counted on a Quebec colony counter; usually only the highest dilution was counted unless the count was too variable or too small.

Mutants.—Five tenths milliliter broth was delivered to each of the 2 sets of 50 small tubes, the contents were mixed and made up to about 5 ml. with melted antibiotic agar cooled to 56° C. The concentration of antibiotic was selected by preliminary experiments under similar conditions so that the bacterial growth was partially, but not completely, suppressed. The 100 tubes were poured into Petri dishes. The tubes were then washed with 5 ml. antibiotic agar which was poured into the same plates. The contents of the plates were then swirled to mix them. The plates were incubated 48 hours at 37° C. and the number of colonies on each of the 100 plates was recorded. The mutation rate was calculated from Formula 1 of Luria and Delbrück[8a, b]

$$a = \frac{-(\ln 2)\ (\ln P_o)}{N} = \frac{(-1.593)\ (\log_{10} P_o)}{N}$$

where a is the mutation rate per bacterium per division cycle, P_o is the proportion of cultures having no resistant mutants, and N is the average number of bacteria per culture at the end of the growth period.

RESULTS

Serial Transfer in Individual Antibiotics.—

Penicillin: The rate of emergence of resistance to penicillin G was measured by serial transfer in broth flasks (Method 1) which contained 100 per cent increases of penicillin on each transfer. Two stock strains of virulent staphylococcus (Nos. 9 and 14) were studied. Beginning with 0.01 unit per milliliter, full growth occurred with each transfer until a concentration of 0.08 unit per milliliter was reached. Above this concentration growth was slower and the

staphylococcus was difficult to maintain on transfer to agar. However, by reducing the increments in penicillin concentration to 0.02 to 0.04 unit per transfer, Strain 9 became resistant to 3.0 units per milliliter after 28 transfers. Strain 14, on the other hand, became resistant to only 0.24 unit per milliliter after 12 transfers (Table I). After developing resistance to penicillin both cultures were made up of tiny colonies on agar resembling "G" variants. They contained only typical gram-positive cocci on smears.

TABLE I. VARIATION IN RATE OF EMERGENCE OF RESISTANCE TO PENICILLIN G IN 7 STRAINS OF STAPHYLOCOCCUS TRANSFERRED SERIALLY IN BROTH FLASKS

STRAIN NO.	PIGMENT	COAGULASE	DNASE*	MAXIMUM PENICILLIN RESISTANCE†	
				UNITS/ML.	NO. OF TRANSFERS
2	++	+	++	5	9
9	+	+	++	3	28
12	±	−	±	0.36	8
14	±	−	−	0.24	12
19	−	−	−	10,000	35
23	++	+	++	55	19
28	−	+	+	35	18

*Desoxyribonuclease activity (see Reference 13).
†As determined by Broth Method 1.

Using the same method, 5 other penicillin-susceptible strains (Nos. 2, 12, 19, 23, and 28) were made resistant (Table I). These strains differed considerably in pigment formation, coagulase activity, and DNAse activity. The rate at which resistance to penicillin was induced was generally slow and the degree of final resistance was limited in most strains. Penicillin resistance was not correlated with pigmentation or enzyme activity. However, after resistance to penicillin appeared, some strains (Strains 2 and 12) did not grow vigorously and on agar the colonies were small, perhaps limiting acquisition of further resistance.

Neomycin: Twelve strains of coagulase-positive *Staph. aureus* were exposed to increasing amounts of neomycin in broth by Method 1. The strains were Nos. 2, 9, 12, 14, 18, 19, 23, 24, 26, 28, 31, and 32. They were selected because all were virulent, antibiotic-susceptible strains, with the exception of Strain 32, which was resistant to penicillin, streptomycin, and the tetracyclines, though susceptible to neomycin. All the strains but Nos. 19 and 24 showed rapid induction of resistance to neomycin as the concentration was increased stepwise by tenfold increments from 0.01 to 1.0 meg. per milliliter, then by twofold steps to 512 meg. per milliliter. There was no change in the morphogenesis, staining, or growth rates as these strains adapted to neomycin. Strains 19 and 24 grew slowly after they had become adapted to 8 meg. per milliliter and only a few colonies were induced to grow in a concentration of 32 meg. per milliliter. Smears of the resistant colonies showed only gram-positive cocci. There was no evidence of induction of cross resistance to penicillin when the neomycin-resistant cultures were exposed to penicillin in broth. The antibiotic-resistant Strain 32 became adapted to neomycin at the same rapid rate as did the majority of the strains tested. There was no apparent relation between pigment production, coagulase activity, or DNAse activity and the rate at which resistance to

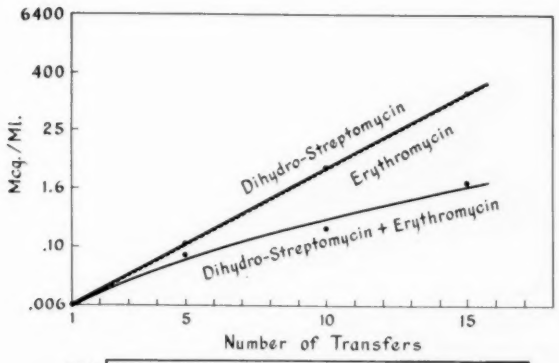


Fig. 1.

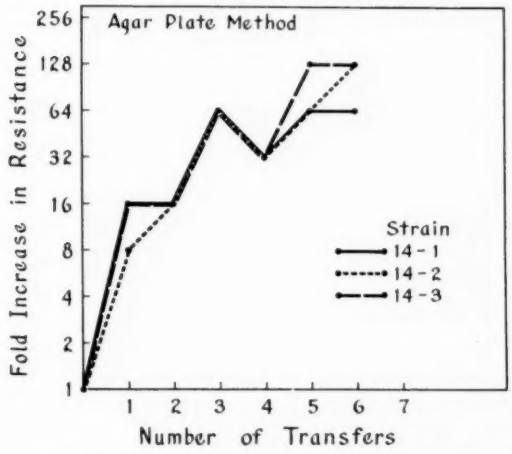


Fig. 2.

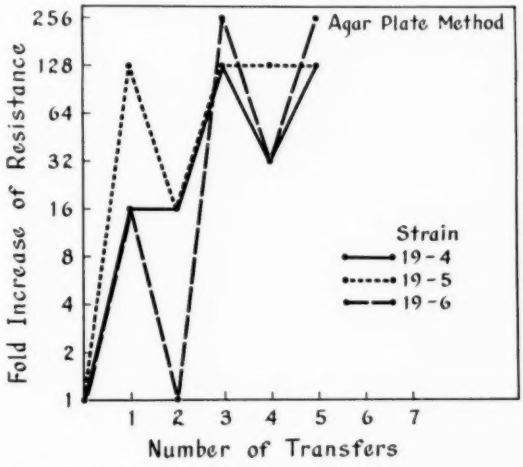


Fig. 3.

Rapid emergence of resistance in *Staph. aureus* to dihydrostreptomycin after serial transfers.

Fig. 1.—Strain 14, serial transfer in broth flasks (Method 1).
Fig. 2.—Three clones from Strain 14, agar plates (Method 3).
Fig. 3.—Three clones from Strain 19, agar plates (Method 3).

neomycin became manifest. With the exception of Strain 28, the majority of the strains which became resistant rapidly possessed good enzyme activity and pigment production. Of the 2 strains which adapted poorly to increasing concentrations of neomycin, Strain 19 lacked enzyme activity and pigment, but Strain 24 was bright yellow and a potent source of both coagulase and DNAse.

Dihydrostreptomycin: Resistance to dihydrostreptomycin was induced in 2 strains of staphylococci (Nos. 9 and 14) by serial transfer in flasks of broth (Method 1). By exposure to doubling concentrations of the antibiotic, resistance increased from 0.01 to 160 mcg. per milliliter in 15 transfers (Fig. 1). The resistant bacteria were unchanged in morphogenesis, staining, and growth on agar and broth media.

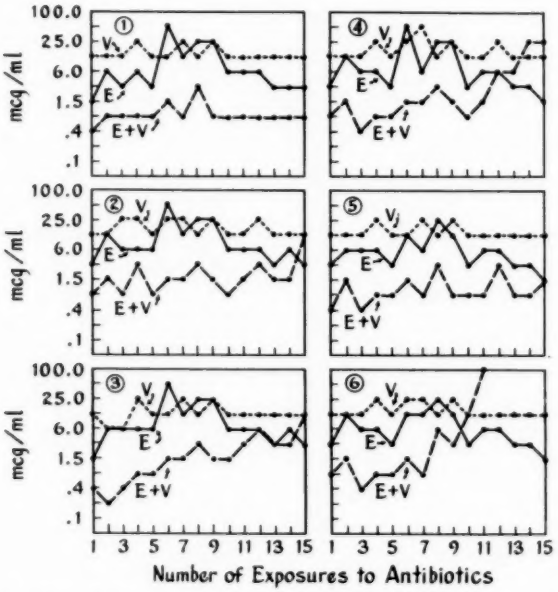


Fig. 4.—The rate of emergence of resistance to erythromycin, vancomycin, and a mixture of equal weights of erythromycin and vancomycin in 6 clones of Strain 14 *Staph. aureus* as demonstrated by serial transfer on agar plates (Method 3).

Resistance to dihydrostreptomycin was also induced by repeated exposure to doubling dilutions of the antibiotic on agar plates (Method 3). Six cultures were obtained from 3 clones each from 2 strains (Nos. 14 and 19). The 6 cultures were all resistant to 0.78 mcg. per milliliter of dihydrostreptomycin before resistance was induced. Complete resistance to 100 mcg. per milliliter was manifest by Strain 14 after 6 transfers (Fig. 2), and in Strain 19 in 5 transfers (Fig. 3). The rate at which the 3 sublines of Strain 14 became resistant was very similar. The rather uniform results suggested that the incidence of resistant mutants was nearly identical in the clones selected. Variations in results arising from inadequate sampling and unselected spontaneous mutations were reduced by transferring only fully grown colonies directly to antibiotic agar without passage through antibiotic-free media. There was considerable

variance and fluctuation in the resistance of the three sublines of Strain 19 to dihydrostreptomycin. The possibility of mutations from resistance to susceptibility was evident, particularly on the second and fourth transfers of Strain 19 (Fig. 3).

Vancomycin: Resistance to vancomycin was induced in two strains, Nos. 9 and 14, by the broth-flask method (Method 1). Strain 9 readily tolerated serial twofold increases in vancomycin from 0.01 to 5.0 meg. per milliliter, but resistance was further increased up to 22 meg. per milliliter only by slow arithmetic increases, requiring 18 transfers in all. Strain 14 became resistant more readily, reaching a level of 60 meg. per milliliter after 13 transfers in doubling concentrations of vancomycin before its growth became slow and erratic.

Strain 14 was also made resistant to vancomycin on agar plates (Method 3). Six clones were selected and made resistant simultaneously. The original isolates were resistant to 12.5 meg. per milliliter in agar. Surprisingly, none of the 6 sublines could be induced to grow in 25 meg. or more per milliliter by this method in spite of 14 transfers (Fig. 4). Agar reduced the activity of vancomycin against staphylococci but it did not increase the rate at which resistance developed.

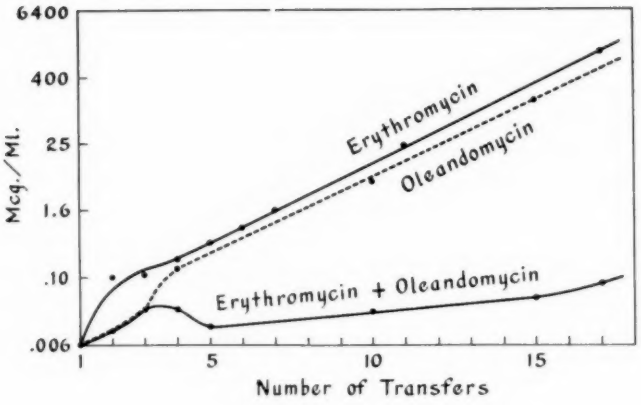
Erythromycin: Strains 9 and 14 were also adapted to erythromycin in flasks of broth (Method 1). By this means both cultures manifest a remarkably rapid adaptation to erythromycin, tolerating an increase from 0.01 to 1,600 meg. per milliliter in 15 successive transfers in (usually) doubling concentrations without any difficulty nor any evident change in growth or staining characteristics (Figs. 1 and 5).

In an attempt to ascertain the affect of resistance to the closely related antibiotic, oleandomycin, upon the rate of adaptation to erythromycin, Strain 11 (naturally resistant to oleandomycin, 100 meg. per milliliter) and a subline of Strain 14 adapted to oleandomycin (80 meg. per milliliter) were made resistant to erythromycin by Method 1. As expected, both strains readily adjusted to increasing amounts of erythromycin in broth, their tolerance jumping from 0.01 to 1.28 meg. per milliliter in 7 steps.

It was assumed that the rate at which staphylococci might be adapted to erythromycin or other antibiotics would be proportional to the size of the population exposed to the selective effect of the antibiotic. To test this hypothesis, Strain 14 was grown in broth which contained doubling dilutions of erythromycin (Method 2) and inoculated with either a 1:100 or 1:10 broth dilution of an 18 hour broth culture. With the smaller population, Strain 14 increased in resistance from 0.39 to only 0.78 meg. per milliliter in 4 transfers (Fig. 6). As expected, with the large inoculum resistance developed more rapidly, increasing from 0.39 to 6.25 meg. per milliliter in 4 transfers (Fig. 7). The level to which resistance had developed, however, was the same after 6 or more transfers with either a small or large bacterial population.

As shown in Table II and Fig. 10 the rate at which erythromycin resistance developed varied with different strains. Two strains, Nos. 24 and 25,

Fig. 5.



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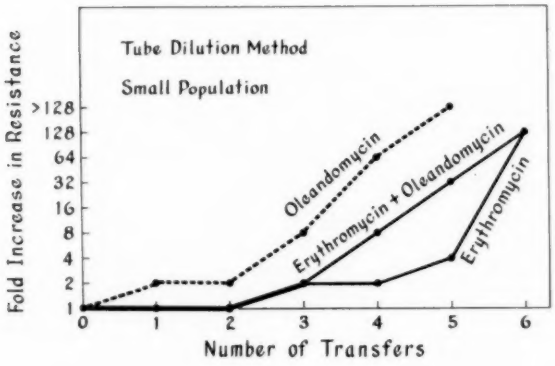

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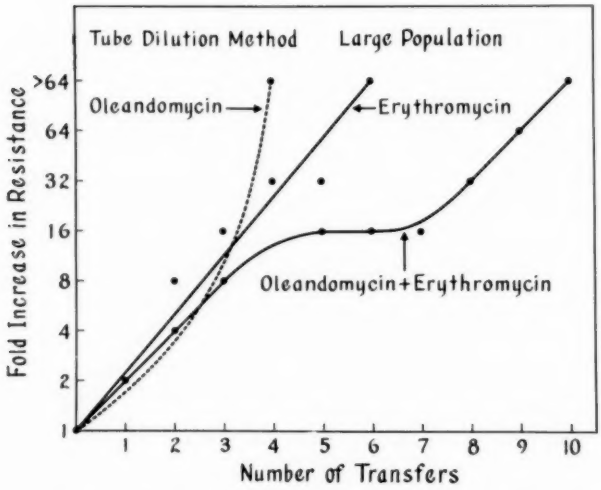


Rates of emergence of resistance of *Staph. aureus* to erythromycin under various conditions.

Fig. 5.—Strain 14, broth flasks (Method 1).

Fig. 6.—Strain 14, tube dilution (Method 2), small population.

Fig. 7.—Strain 14, tube dilution (Method 2), large population.

with the greatest amount of pigment, coagulase and DNAse activity became resistant most rapidly. Though one strain (No. 19) with little or no pigment or enzyme activity developed no resistance, 3 similar strains became slowly resistant to 50 to 100 meg. per milliliter. After the sixth transfer on erythromycin agar, all 6 strains grew somewhat erratically on antibiotic-free agar. The apparent "dependence" on erythromycin was only partial and was most frequent with Strain 25, which never reached a very high level of resistance. The resistant progeny of Strain 25 grew poorly on all plates.

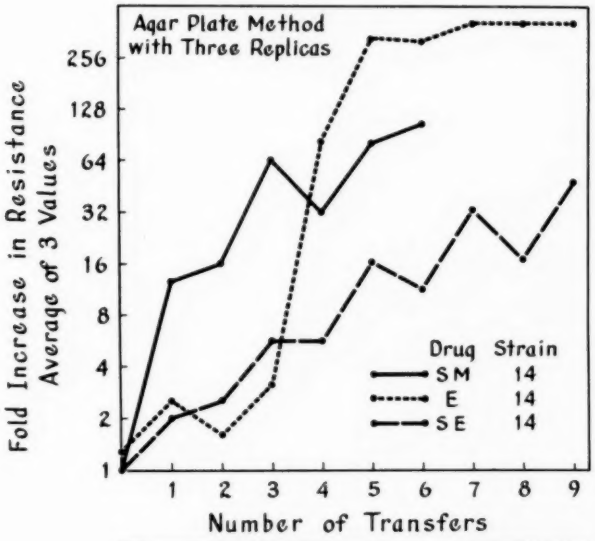


Fig. 8.

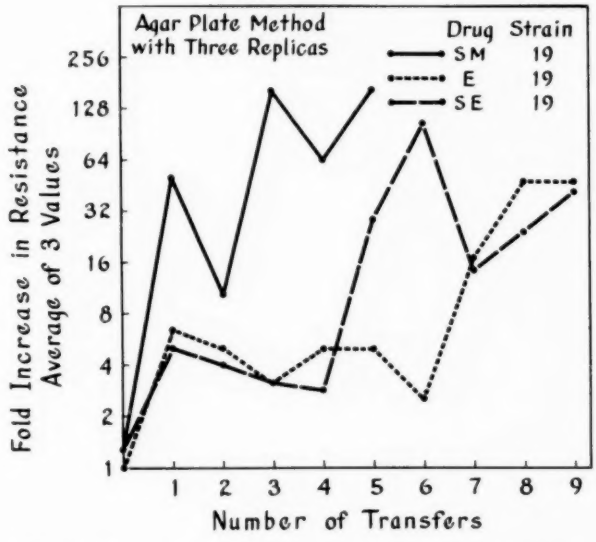


Fig. 9.

Fig. 8.—Strain 14, agar plates (Method 3), average of 3 replicas with erythromycin *(E)*, streptomycin *(SM)*, and both drugs *(SE)*.

Fig. 9.—Strain 19, agar plates (Method 3), average of 3 replicas with erythromycin *(E)*, streptomycin *(SM)*, and both drugs *(SE)*.

TABLE II. RATE OF EMERGENCE OF RESISTANCE TO ERYTHROMYCIN IN 6 STRAINS OF STAPHYLOCOCCUS TRANSFERRED SERIALLY ON AGAR PLATES

STRAIN NO.	PIGMENT	COAGULASE	DNASE*	MAXIMUM ERYTHROMYCIN RESISTANCE†	
				MCG./ML.	NO. OF TRANSFERS
3	±	–	–	100	9
12	±	–	±	50	9
14	±	–	–	50	9
19	–	–	–	0.78	9
24	+++	+	+++	100	5
25	++++	+	++	6.25	4‡

*Desoxyribonuclease activity (see Reference 13).
†As determined by Agar Plate Method 3.
‡No more resistant after 9 transfers but partially "dependent."

The fact that the rate of adaptation to erythromycin was variable in staphylococci was especially striking in another experiment with two strains, Nos. 14 and 19. Three clones were picked from each and made resistant by serial transfer on erythromycin-agar (Method 3). On the whole, Strain 14 became resistant to erythromycin more rapidly than did Strain 19 (Figs. 8 and 9). Figs. 11 and 12 demonstrate, however, that there was considerable difference in the level of resistance reached by the sublines of each strain, especially in Strain 14 after 3 or more transfers and Strain 19 after 7 or more exposures.

Since the variation noted above might be the result of sampling errors, the experiment was repeated with modifications designed to decrease such variations. Six clones of Strain 14 were selected and simultaneously adapted to erythromycin on agar plates by Method 3. In this case each transfer was made from the plate which contained the highest concentration of erythromycin permitting *any* growth rather than from the concentration allowing *full* growth. In this instance there was striking similarity in the rate at which resistance became apparent in each of the 6 sublines (Fig. 4). There was no more than a 2 dilution difference (200 per cent) at any time. Initially the clones were resistant to 1.56 to 3.12 meg. per milliliter of erythromycin. After 5 transfers 4 sublines reached a peak resistance of 50 meg. per milliliter whereas 2 grew only in 12.5 meg. per milliliter. Resistance remained essentially the same after 9 exposures to erythromycin but declined thereafter, reaching a level of 1.56 to 3.12 meg. per milliliter after 15 exposures. This loss of resistance was consistent in all 6 sublines and was not accompanied by any apparent antibiotic dependence or decline in vigor of growth.

Erythromycin is a polyhydroxyketolactone that contains, for the most part, erythromycin A and a small amount of the similar erythromycin B, which is 15 to 20 per cent less active.[9]

Six clones of Strain 14 were adapted to erythromycin A, to erythromycin B, and to a mixture of equal parts of A and B by the agar plate dilution method (Method 3). The powdered antibiotic bases were dissolved in absolute methyl alcohol and the dilutions were made with phosphate buffer solution, pH 8.0.[9] On the initial test the 6 clones were susceptible to 0.2 meg. per milliliter

erythromycin A, 0.39 erythromycin B, and 0.39 erythromycin A plus B (Fig. 13). Serial transfer was made of whatever colonies appeared in the highest concentration of antibiotic which permitted any growth. Subline 1 became fully resistant to 100 mcg. per milliliter of each of the 3 antibiotic preparations after only 6 exposures though the other 5 sublines remained susceptible to 0.78 to 12.5 mcg. per milliliter. Sublines 3 and 5 became completely resistant to erythromycin B after 7 exposures but remained susceptible to erythromycin A; subline 3 was resistant to A plus B and subline 5 was susceptible to A plus B. After the seventh exposure subline 6 was resistant to 25 mcg. per milliliter A plus B though it remained susceptible to both A and B individually. After 8

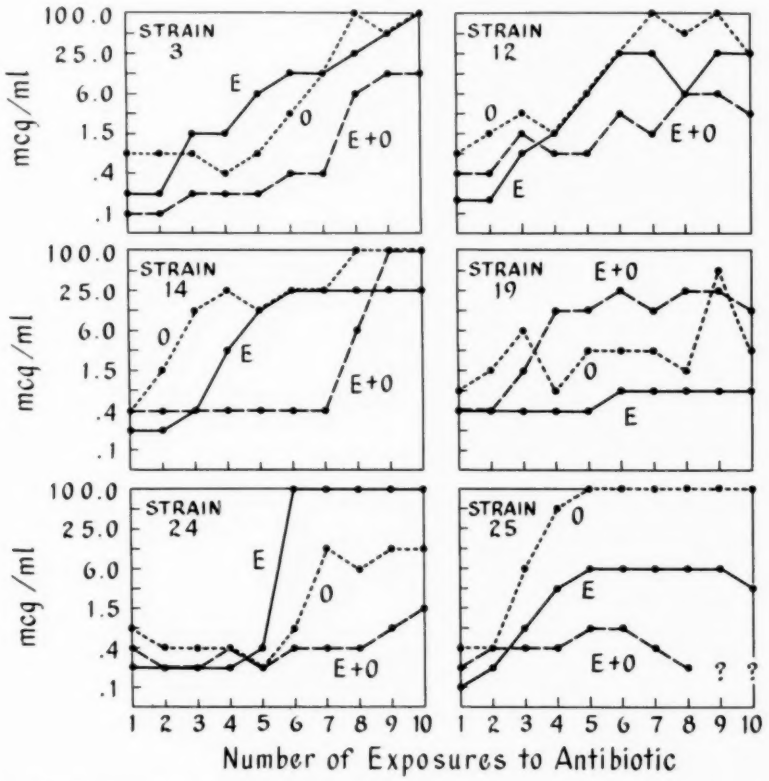


Fig. 10.—Variation in rate of adaptation (agar plates, Method 3) to erythromycin, oleandomycin, and a mixture of erythromycin plus oleandomycin in 6 strains of *Staph. aureus*.

exposures all but subline 2 were fully resistant to A plus B 100 mcg. per milliliter though most remained susceptible to A in varying degrees and 3 sublines (2, 4, and 6) continued susceptible to B. Sublines 1 and 6 became fully resistant to A by the sixth and ninth exposure, respectively. The experiment was terminated after 12 transfers. Tests for cross resistance showed that 3 sublines adapted to erythromycin B had become somewhat resistant to A. Four sublines adapted to A had become cross resistant to even greater concentrations of the less active erythromycin B.[10]

Oleandomycin: One of the newer antibiotics, oleandomycin, is very similar to erythromycin, though somewhat less active against staphylococci and other bacteria.[10] Two strains (Nos. 9 and 14) of staphylococci were made resistant to oleandomycin by serial transfer in flasks of broth (Method 1). Both strains were readily adapted to an increase in the concentration of oleandomycin from 0.01 to 160 meg. per milliliter in 14 twofold steps (Fig. 5). A subculture of

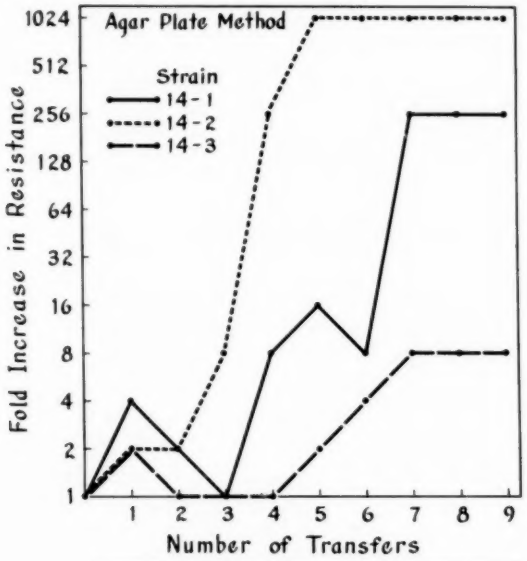


Fig. 11.

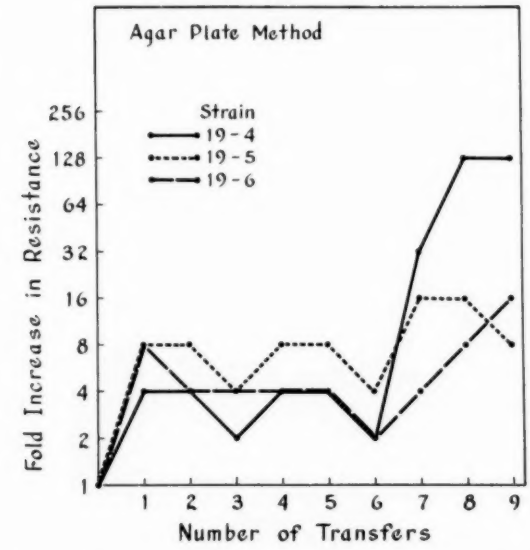


Fig. 12.

Variation in rate of emergence of resistance to erythromycin between clones of *Staph. aureus.*

Fig. 11.—Strain 14, agar plates (Method 3), 3 clones.
Fig. 12.—Strain 19, agar plates (Method 3), 3 clones.

Strain 14, which had been adapted to erythromycin (100 meg. per milliliter), remained fully susceptible to oleandomycin, though it could be made quickly resistant to it by Method 1.

During serial transfer in broth dilutions (Method 2) of oleandomycin, Strain 14 became adapted to the antibiotic somewhat more rapidly than it had with erythromycin (Fig. 6). When the experiment was repeated with a tenfold greater population, resistance to oleandomycin developed even more rapidly, as was the case with erythromycin (Fig. 7).

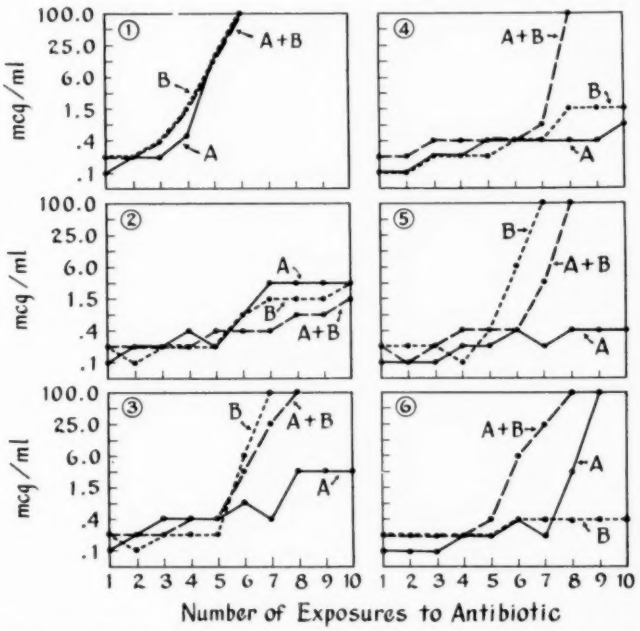


Fig. 13.—The rate of emergence of resistance to erythromycin A, erythromycin B, and a mixture of equal weights of erythromycins A and B in 6 clones of Strain 14 *Staph. aureus* during serial transfer on agar plates (Method 3).

As with erythromycin, 6 strains of staphylococcus were adapted to oleandomycin by serial transfer on agar plates which contained doubling dilutions of the antibiotic (Method 3). As shown in Fig. 10, all the strains were initially more susceptible to erythromycin than to oleandomycin. With one exception (Strain 24), the cultures remained more susceptible to erythromycin than to oleandomycin after adaptation. The rate and degree to which resistance to oleandomycin became manifest varied considerably with the 6 strains. The speed with which resistance developed to erythromycin was approximated in the case of oleandomycin; the differences seemed to be dependent more on the staphylococcal strains than upon the slight difference in the activity and structure of the 2 antibiotics.

Serial Transfer Data With Antibiotics in Pairs.—

Oleandomycin plus erythromycin: Two strains (Nos. 9 and 14) of staphylococci were transferred serially in flasks of broth (Method 1) which contained

equal weights of erythromycin and the similar antibiotic, oleandomycin. The 2 very susceptible strains had adapted readily to these antibiotics separately but surprisingly great difficulty was encountered in making them resistant to both antibiotics simultaneously by this method. Strain 9 failed to grow on transfer in doubling concentrations. After several transfers at 0.02 meg. per milliliter the organism adapted to serial arithmetic increases in concentration of the 2 antibiotics, resistance increasing from 0.01 to 0.16 meg. per milliliter in 12 transfers and then increasing by doubling steps without any difficulty. Strain 14 grew in 0.01 meg. per milliliter total concentration of the 2 antibiotics and only after several unsuccessful attempts was it trained to multiply in 0.04 meg. per milliliter. Thereafter Strain 14 was easily adapted to successive twofold increases in antibiotic concentration (Fig. 5).

Our next concern was with the effect of resistance to one of these antibiotics upon the rate at which resistance might be induced to a mixture of erythromycin and oleandomycin. Strain 11, a staphylococcus naturally resistant to oleandomycin though fully susceptible to erythromycin, was easily adapted to successive doubling concentrations of erythromycin by Method 1. By the same technique, its resistance to erythromycin plus oleandomycin was quickly increased from 0.01 to 1.28 meg. per milliliter in 7 twofold steps. Likewise subcultures of the susceptible Strain 14, which had been separately adapted to either oleandomycin or erythromycin, were quickly made resistant to a mixture of the 2 antibiotics.

When the susceptible Strain 14 was exposed to serial broth dilutions (Method 2) of the antibiotic mixture, the result was somewhat different (Fig. 6). If a relatively small population of bacterial cells (1:100 dilution of 18 hour broth culture) was exposed, little resistance developed at first but after 3 transfers resistance developed rapidly to the mixture, the rate being intermediate between that for oleandomycin alone (earliest resistance) and erythromycin alone (late resistance). When a large population (1:10 dilution) was used, resistance developed with equally great rapidity to oleandomycin, erythromycin, and the mixture of the 2 in early transfers but the rate seemed to decline for the mixture after the third transfer (Fig. 7).

Equally susceptible cultures of staphylococci may become resistant to antibiotics at quite different rates. Six strains (Nos. 3, 12, 14, 19, 24, and 25) were made resistant to a mixture of oleandomycin and an equal amount of erythromycin by several transfers on agar plates which contained doubling dilutions of the antibiotic mixture. As shown in Fig. 10, most strains developed resistance to the 2 drugs more slowly than they did to either antibiotic alone. Strains 3 and 14 developed late resistance to the mixture. Strains 12 and 24 showed very modest resistance to the 2 antibiotics together and considerable resistance to each alone. Strain 19 was a curious exception to the general rule becoming more resistant to the mixture than to either antibiotic alone. Strain 25 was not successfully adapted to increasing concentrations of the 2 antibiotics but after the seventh transfer failed to grow in any antibiotic concentration and

thereafter, even the drug-free control plates showed no growth. This and other strains also failed to grow on some control plates after 7 or more transfers on erythromycin agar and rarely after several transfers on oleandomycin agar though true drug dependence was not proved.

Vancomycin plus erythromycin: Resistance to a mixture of equal parts of these 2 antibiotics was induced in 2 strains (Nos. 9 and 14) of staphylococci by Method 1 in flasks of broth. Strain 9 did not adapt readily to doubling concentrations of the mixture but it did grow when the antibiotics were increased by arithmetic increments from 0.01 to 0.16 meg. per milliliter. Thereafter it quickly adapted to doubling increases of the 2 antibiotics. Without delay, Strain 14 became resistant to twofold increases from 0.01 to 40 meg. per milliliter in broth flasks.

TABLE III. MUTATION RATE TO RESISTANCE TO VARIOUS ANTIBIOTICS IN A STRAIN OF STAPHYLOCOCCUS AUREUS (14)

ANTIBIOTICS	CONCENTRATION (MCG./ML.)	MUTATION RATE*
Erythromycin	0.9	2.9×10^{-11}
Erythromycin A	0.8	3.2×10^{-11}
	0.9	2.0×10^{-11}
Erythromycin B	0.9	5.5×10^{-11}
Oleandomycin	6.0	2.2×10^{-12}
	7.5	3.4×10^{-12}
	10.0	8.9×10^{-12}
Vancomycin	20.0	5.0×10^{-11}
Erythromycin + erythromycin A	0.4 + 0.4	9.3×10^{-12}
Erythromycin + erythromycin B	0.4 + 0.4	4.0×10^{-11}
Erythromycin + oleandomycin	0.4 + 4.0	3.4×10^{-11}
Oleandomycin + vancomycin	4.0 + 10.0	6.4×10^{-12}

*Average of 2 to 4 experiments.

This experiment was repeated with 6 clones of Strain 14 using agar plates (Method 3). Only 1 of the sublines became fully resistant to 100 meg. per milliliter vancomycin plus erythromycin, and this resistance developed late and rather abruptly after the ninth transfer (Fig. 4). One subline remained susceptible to 1.56 meg. per milliliter of the mixture after 14 transfers. The other 4 showed slowly developing resistance. In this experiment the presence of the less active vancomycin seemed to retard the emergence of resistance to the mixture of erythromycin and vancomycin. Though early, but temporary, resistance developed to erythromycin alone, no resistance to vancomycin itself became evident in this experiment.

Vancomycin plus oleandomycin: Similarly, resistance was induced in Strains 9 and 14 to a mixture of equal parts of vancomycin and oleandomycin in flasks of broth (Method 1). Both strains were easily adapted to doubling increases of the antibiotics from 0.01 to 20 meg. per milliliter in 11 transfers.

Vancomycin plus dihydrostreptomycin: By the same method (Method 1) Strains 9 and 14 were adapted to vancomycin plus dihydrostreptomycin. Strain 9 grew readily as the concentration was increased by twofold steps from 0.005 to 0.16 meg. per milliliter; after failing to grow at 0.32 meg. per milliliter growth occurred readily as the concentration was increased by 5 arithmetic intervals from 0.08 to 0.64 meg. per milliliter. Strain 14 was adapted to doubling concentrations from 0.01 to 40 meg. per milliliter though growth was slow at 2 stages of adaptation (0.04 and 80 meg. per milliliter).

Fig. 14.

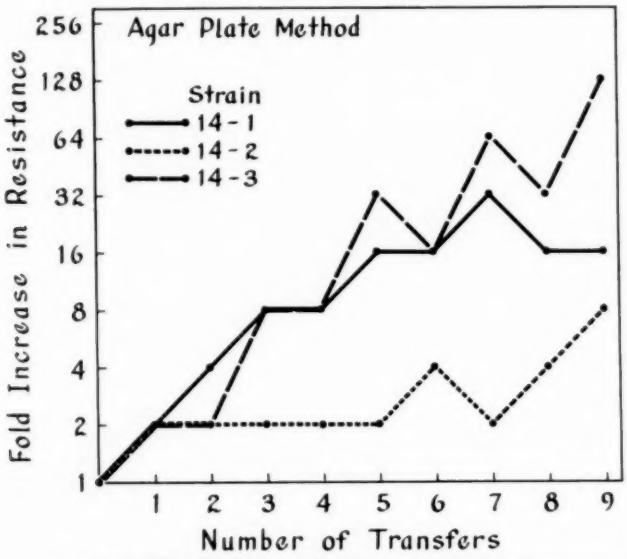
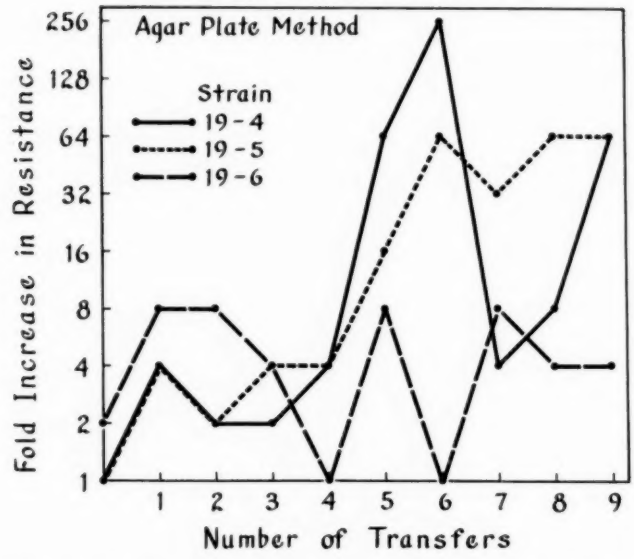


Fig. 15.



Variation in the rate of adaptation of clones of *Staph. aureus* to a mixture of equal weights of erythromycin and streptomycin.

Fig. 14.—Three clones of Strain 14, agar plates (Method 3).

Fig. 15.—Three clones of Strain 19, agar plates (Method 3).

Erythromycin plus dihydrostreptomycin: A mixture of these antibiotics was employed with the same method (Method 1) and strains. Strain 9 grew in 0.005 and 0.0075 meg. per milliliter but on 2 trials failed to grow in 0.01 meg. per milliliter. Strain 14 was induced to grow in doubling amounts from 0.01 to 5.0 meg. per milliliter although it was slow to adapt to an increase from 0.08 to 0.16 meg. per milliliter; the rate of adaptation was slower than for erythromycin or dihydrostreptomycin alone (Fig. 1).

By the plate method (Method 3) clones each from Strains 14 and 19 were made resistant to erythromycin plus dihydrostreptomycin. As shown in Fig. 14, 2 clones of Strain 14 adapted readily but the third did not. The behavior of Strain 19 was erratic (Fig. 15). One clone showed fluctuating resistance at a high level and another varied at a low level of resistance, whereas a third developed a late, stable resistance of a high order.

Oleandomycin plus dihydrostreptomycin: Strain 9 grew in 0.005 meg. per milliliter of oleandomycin and dihydrostreptomycin but was induced to grow in 0.0075 meg. per milliliter only after 3 trials. Thereafter it multiplied quickly as the concentration was increased from 0.01 to 0.64 meg. per milliliter in 6 steps. Strain 14 grew in 0.01 meg. per milliliter and adapted to doubling amounts up to 20 meg. per milliliter.

Mutation Rate to Resistance.—The mutation rate to resistance to various antibiotics in Strain 14 *Staph. aureus* is shown in Table III. The concentration of the antibiotics was chosen by preliminary experiments with the same bacterial inoculum on agar plates so as to suppress growth partially but not completely. The mutation rate of resistance at a bacteriostatic concentration of the antibiotic was in descending order of frequency as follows: erythromycin B (greatest), vancomycin, erythromycin A, and oleandomycin (least). It may be seen that in the case of oleandomycin the mutation rate was not inversely proportional to the concentration, contrary to what one might expect. Furthermore, the mutation rate was not greatly reduced by mixing 2 antibiotics in half their bacteriostatic concentrations, using either similar or dissimilar antibiotics, except in the case of erythromycin plus erythromycin A.

DISCUSSION

The relatively slow rate at which resistance to penicillin can be induced in staphylococci by serial transfer is well known. It is usually attributed to infrequent mutations yielding variants of slight resistance. The increase in resistance to penicillin is, therefore, gradual.[3a, b] The maximum level of resistance attained is often not high, perhaps because the resistant mutants grow slowly and have little vitality. It is also known that resistance, which has been induced with penicillin in staphylococci, is often temporary and is not associated with the induction of penicillinase.[3c]

We found that staphylococci were in most cases quickly adapted to neomycin in high concentrations without any visible alteration in their morphogenesis or growth. This observation was somewhat surprising. Neomycin has been used extensively in this and other hospitals for several years, yet resistance has

rarely been manifest in staphylococci isolated from patients. This fact might be the result of poor absorption of neomycin from the intestinal tract. It should be noted, however, that fecal staphylococci are seldom resistant to neomycin, even after neomycin is given orally for several days. It seems likely that the high fecal concentrations of the bactericidal neomycin may account for the infrequent resistance in intestinal staphylococci.

Most workers have found that staphylococci can be quickly adapted to high concentrations of streptomycin. This type of resistance in bacteria is so common with the antibiotic that it has been called the "streptomycin type." An infrequent single step mutation to a very high level of resistance is generally thought to be the responsible mechanism. However, in the experiments reported here only one strain (No. 14) demonstrated uniform resistance of this type, whereas another strain (No. 19) showed fluctuating resistance to dihydrostreptomycin during serial transfers.

Resistance to vancomycin has been difficult to induce in staphylococci. Two strains transferred in broth showed slow adaptation in one case and more rapid but limited resistance in the other. No significant degree of resistance was induced in the latter strain during transfers on vancomycin agar plates in spite of the fact that agar appeared to lessen the activity of the antibiotic. The finding of limited induction of resistance of vancomycin is supported by the fact that staphylococci have not been found to become resistant during treatment with vancomycin.

Erythromycin is one of the more effective antibiotics used in staphylococcal infections. Unfortunately, susceptible staphylococci have often been replaced by resistant organisms during therapy, particularly during the treatment of open lesions in hospitals where the antibiotic has been used frequently. In 1958 we found 21.6 per cent of 471 strains of pathogenic staphylococci isolated from infected lesions in our hospital were resistant to erythromycin. This is about the same frequency which has been observed for the past 5 years. Most strains of staphylococci are easily and quickly adapted to high concentrations of erythromycin in vitro. This is particularly true with strains which are resistant to oleandomycin or other antibiotics similar in chemical structure to erythromycin.

The rate at which resistance to erythromycin could be induced in staphylococci appeared to be partially dependent upon several properties of the bacteria. These included the size of the bacterial population, pigment production, coagulase and DNAse activity. The degree of erythromycin resistance which was induced on agar plates appeared to be limited by the occurrence of a partial dependence upon erythromycin, resulting in a slower rate of growth. The rate of development of resistance to erythromycin seemed to vary among different clones in some strains of staphylococci, a variation which seemed to be related in part, at least, to sampling variations inherent in the induction method. After many transfers on erythromycin agar, one strain (No. 14) was observed to become less resistant, perhaps as the result of reverse mutations.

The exact constituents of a preparation of erythromycin may also play a role in the rate at which erythromycin resistance may be induced in staphylococci. Thus, resistance to erythromycin B was more readily induced than to the more active erythromycin A. Resistance was easily induced to a mixture of equal parts of erythromycins A and B, perhaps because the mixture had less activity than did erythromycin A alone. Cross resistance to erythromycins A and B undoubtedly was partly responsible for the rapid resistance induced by the mixture of the 2.

Resistance was readily induced with oleandomycin and there was no dependence or return to susceptibility. Though closely related in structure, oleandomycin did not induce cross resistance in all strains of staphylococci to erythromycin. Perhaps because it is more active, erythromycin usually induced cross resistance to oleandomycin. Occasionally staphylococci isolated from patients may be highly resistant to the one antibiotic and not to the other. Cross resistance was easily induced in such strains, hence the absence of cross resistance does not mean that adaptation is impossible.

The main objective of the present study was to see if the addition of 2 antibiotics in mixtures of equal concentration would retard adaptation of staphylococci to them. It is generally held that resistance is less likely to occur if 2 dissimilar antibiotics are administered simultaneously. The use of oleandomycin in such mixtures has been discouraged, because it might induce cross resistance to the more active related antibiotic, erythromycin. It was rather surprising therefore that in our experiments resistance appeared only after some delay in staphylococci exposed to oleandomycin and erythromycin in flasks of broth. Staphylococci that were resistant to either oleandomycin or erythromycin were easily adapted to the mixture of the 2, which indicates that delay in adaptation occurs only when both drugs are independently active in retarding growth. Delay of adaptation to mixtures of the 2 similar antibiotics could be shown in doubling broth dilutions of antibiotic if relatively small numbers of staphylococci were exposed. This was not true with large populations, perhaps because mutations to double drug resistance were more frequent. The considerable differences in the rate at which different strains of staphylococci became resistant to oleandomycin plus erythromycin appear to be the result of marked inherent differences in their capacity to resist. This capacity varied from an inability to adapt to an enhancement of resistance in the presence of the 2 antibiotics. The delay in resistance in some cases appeared to be, at least in part, the result of a frequent failure to grow in the absence of the antibiotics after several transfers under their influence.

Inhibition of the adaptation phenomenon was more easily demonstrated with combinations of quite dissimilar antibiotics. Thus the addition of the less active antibiotic, vancomycin, to erythromycin significantly delayed resistance in staphylococci to erythromycin. Perhaps the result reflects the rather slight capacity of staphylococci to adapt to vancomycin per se. Oddly, vancomycin failed to retard resistance to oleandomycin. Vancomycin added

to dihydrostreptomycin delayed adaptation at some concentrations, but once staphylococci were adapted at these levels resistance could usually be rapidly induced.

A combination of erythromycin with dihydrostreptomycin gave somewhat erratic results. Resistance was retarded with some strains and some concentrations but not with others. Others have reported more success in delay of resistance with this combination.[11] The original susceptible strains showed less capacity to grow in the antibiotic mixture than with either drug alone. Similar results were obtained with oleandomycin plus dihydrostreptomycin.

The studies of mutation rates to double drug resistance can be regarded as being only preliminary since only one strain of staphylococcus and a limited number of antibiotics were used. The experience of Treffers[12] was confirmed, in that mutation rates could be determined quite precisely, whereas transfer data gave only a crude index of the capacity of the staphylococci to adapt to antibiotics. Mutation rates are not difficult to determine, but the size of the bacterial population must be carefully adjusted and the mutation rate in any one experiment is known only for the one (bacteriostatic) concentration of antibiotic which one chooses to test. The calculation of the mutation rate has been the subject of some controversy. We have chosen to use the rather simple "negative tube" procedure (Formula 1) of Luria and Delbrück.[5a, b]

Strangely, the mutation rate to resistance was not found to be directly proportional to the concentration of oleandomycin. This might reflect some error in the method but could also reflect the characteristics of the bacterial strain and the antibiotic. Only in the case of erythromycin plus erythromycin A was the mutation rate lower for antibiotic pairs than for the individual antibiotics. This aspect of the problem deserves further study. If the observation were confirmed, one might question the modus operandi of antibiotic combinations in patients with staphylococcal infections, particularly since there is little evidence of synergistic enhancement of growth inhibition in vitro with such combinations.[6, 10]

Very little is known as to the effect of antibiotic combinations upon the adaptation of staphylococci to antibiotics in human beings. Lepper and others[1] administered spiramycin and novobiocin simultaneously to a group of patients in a hospital over a period of 6 months beginning 4 months after a period in which spiramycin had been used alone. Nose and throat cultures were examined for staphylococci and appeared to show a delay in resistance for only about 2 months with the antibiotic combination. Previous use of the closely related erythromycin, however, may have reduced the effectiveness of the less active spiramycin.

Barber and co-workers[2] used antibiotic combinations for 6 months in a large hospital. Susceptibility tests were performed on the staphylococcal isolates. Evidence of cross resistance to erythromycin and chloramphenicol was unexpectedly found. There was no evidence of cross resistance between erythromycin and novobiocin, a combination favored by the authors.

SUMMARY

1. The rate of emergence of resistance to various antibiotics was measured for 15 strains of virulent staphylococci. Three methods were employed: serial transfer in flasks of antibiotic broth, transfer in doubling dilutions of antibiotic broth tubes, and transfer on agar plates containing the antibiotics. The antibiotics were tested either singly or in paired combinations and included penicillin, dihydrostreptomycin, erythromycin, oleandomycin, neomycin, and vancomycin. Mutation dates for antibiotic resistance were also measured by the negative-tube procedure of Luria and Delbrück.

2. Transfer data gave a crude measure of the capacity of staphylococci to develop resistance to antibiotics. The results were influenced by such variables as the concentration of antibiotic, the activity of the antibiotic, the medium employed, size of the bacterial population, time of bacterial exposure to the antibiotic, bacterial sampling techniques, variation between bacterial strains, and variation in bacterial clones within a strain.

3. Resistance to penicillin developed slowly on transfer and reached a moderate level. It was limited by the appearance of small colony variants which grew slowly on laboratory media. Most strains of staphylococci were quickly adapted to high concentrations of neomycin and dihydrostreptomycin without any change in morphogenesis, staining, or growth rate. Resistance to vancomycin was induced quite readily in one strain in broth but only slowly in another strain. No resistance to vancomycin emerged during transfer on agar plates. Adaptation to erythromycin occurred quickly with some virulent strains of staphylococci but not with others. Some strains of staphylococci grew erratically on agar plates after several transfers on erythromycin agar, although complete antibiotic dependence was not evident. Variations in the rate of adaptation to erythromycin were also shown between clones within a virulent strain. Furthermore, the rate at which resistance developed to erythromycin A was often quite different than for the less active erythromycin B. Oleandomycin-resistant strains quickly became adapted to erythromycin, to which antibiotic oleandomycin is chemically related. Most strains of susceptible staphylococci became quickly resistant to oleandomycin on serial transfer.

4. The combination of equal weights of erythromycin and oleandomycin delayed the emergence of resistance in most strains of staphylococci upon serial transfer. Vancomycin also retarded adaptation of staphylococci to erythromycin, as expected. Vancomycin also seemed to delay resistance to dihydrostreptomycin but it had no such effect on resistance to oleandomycin. Resistance to erythromycin plus dihydrostreptomycin usually developed at a slower rate than to either drug alone. Oleandomycin-dihydrostreptomycin mixtures delayed the emergence of resistance on serial transfer but the rate of adaptation was rapid after a few transfers.

5. Preliminary studies of mutation rates to resistance to antibiotics with a virulent strain of staphylococcus had some surprising results. The mutation rate was not closely related to the rate at which resistance emerged on serial transfers. At bacteriostatic concentrations of antibiotics the mutation rate was

inversely proportional to the concentration required to inhibit staphylococcal growth. Mixtures of the antibiotics in half the minimal bacteriostatic concentration did not markedly reduce the mutation rate, except in the case of erythromycin plus erythromycin A.

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EFFECT OF HEMORRHAGIC SHOCK UPON THE PROPERDIN SYSTEM OF THE DOG

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The adverse effect of shock on immune responses was studied by the measurement of complement and phage neutralization titers in the serum of dogs subjected to standardized hemorrhagic shock. Phage neutralization is believed to be dependent on the properdin system. Complement levels were not appreciably affected. No significant change in phage neutralization titer was observed during the oligemic phase of shock, but during the posttransfusion period these levels declined progressively to reach values just prior to death of about one half of the control levels.—ED.

RECENT studies have demonstrated that animals subjected to hemorrhage or other shock inducing procedures have a lowered resistance to certain bacteria and toxins.[1] A possible correlate of this lowered resistance is a reduction in the plasma protein, properdin, a factor which may play an important role in natural immunity.[2] Using the zymosan assay method, Frank and co-workers[3] have reported a significant reduction in properdin levels after hemorrhage.

An alternative method for studying this immunologic mechanism has been developed on the basis of bacteriophage neutralization.[4] Fresh sera can inactivate T2-phage infections of *Escherichia coli*. A complete analysis of all the factors responsible for this inactivation has not been worked out, but it has been demonstrated[5, 6] that phage neutralization is dependent upon the properdin system defined by Pillemer and co-workers.[2] Although it must be recognized that there is still some question as to whether the phage neutralization technique assays the identical component as that of the zymosan technique,[4] the phage neutralization method has several distinct advantages: (a) it is more sensitive to changes in properdin concentration; (b) it is not affected by the levels of heparin normally used as an anticoagulant in hemorrhagic shock studies; and (c) it is more readily inhibited by certain lipopolysaccharides of mammalian origin.[6] In the present study, changes in the properdin system in the sera of dogs subjected to a standardized bleeding procedure were investigated with the use of the phage neutralization technique.

MATERIALS AND METHODS

Hemorrhagic shock was produced in 6 unanesthetized dogs by a procedure similar to that employed by Frank and collaborators.[3] Each animal was premedicated with morphine

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(2 mg. per kilogram). One pair of femoral vessels was cannulated under local procaine anesthesia, with aseptic precautions. Heparin was administered intravenously to prevent clotting. Mean arterial pressure was measured by a damped mercury manometer.

After a control period of 15 to 20 minutes, the animal was bled into a sterile graduated cylinder until a mean blood pressure of 30 mm. Hg was attained. It was maintained at this level by means of a pressure regulator which has been previously described.[7] The volume of blood within the reservoir was recorded at frequent intervals. Characteristically, this volume would increase for a certain period of time, after which the animal would begin to take blood back from the chamber. The reservoir was swirled gently at frequent intervals to prevent settling of the erythrocytes. After 40 per cent of the maximum bleeding volume had returned spontaneously to the animal, the remaining blood was rapidly reinfused. Blood pressure was then observed for several hours, usually until the death of the animal.

Two arterial blood samples were withdrawn, 10 minutes apart, during the control period. Subsequent samples were taken at intervals of approximately 1 hour during the oligemic phase of the experiment. A sample was withdrawn immediately upon completion of the rapid transfusion of the remaining 60 per cent of the maximum bleeding volume, and at approximately 1 hour intervals thereafter. A final sample was obtained just prior to death.

Two additional experiments were conducted to control the effects of morphine. Blood samples were obtained at intervals of approximately ½, 4, and 7½ hours after the subcutaneous injection of morphine sulfate (2 mg. per kilogram). These animals were subjected to no blood loss other than the negligible volumes removed for analysis.

All blood specimens were centrifuged immediately after collection. The plasma was separated and kept at 0° C. until after the completion of the experiment. They were then coded and placed in a Deepfreeze until the assays for phage neutralization and complement could be carried out.

The phage neutralization titer was assayed by the method previously described.[4] The activity is expressed in terms of 50 per cent neutralizing units per milliliter of undiluted plasma ($Ph.N_{50}$). Complement was measured by the method of Osler and associates,[8] and is expressed in terms of 50 per cent hemolytic units per milliliter of undiluted plasma ($C''H_{50}$).

RESULTS

The hemodynamic responses to hemorrhage of the 6 animals in this series were quite similar to those previously reported in animals subjected to the same shock inducing procedures.[9] After the rapid initial hemorrhage, the bleeding volume continued to increase gradually until the maximum volume was attained after an interval of 69.8 ± 28.6 minutes (mean ± S.D.). The maximum bleeding volume averaged 63.1 ± 9.5 ml. per kilogram body weight. A gradual return of blood from the reservoir followed. Forty per cent of the maximum bleeding volume was taken back by the animal in 211 ± 87 minutes after the inception of hemorrhage in 5 animals. The sixth animal (represented by the solid triangles in Fig. 1) died suddenly 296 minutes after the beginning of hemorrhage. At this time, the volume of blood in the reservoir was still 84 per cent of the maximum value.

The hematocrit ratio tended to diminish early in the bleeding period. Toward the end of the bleeding period, however, it began to rise, and continued its ascent during the posttransfusion phase. Thus, the hematocrit level rose from a mean control value of 46.5 ± 5.3 per cent to a preterminal value of 58.2 ± 5.4 per cent.

The control mean arterial pressure prior to hemorrhage averaged 109 mm. Hg (range, 95 to 125 mm. Hg). The changes in blood pressure after rapid transfusion of the remaining 60 per cent of the maximum bleeding volume are illustrated in the upper right quadrant of Fig. 1. In 4 of the experiments, there was a progressive decline in pressure, with death ensuing an average of 163 minutes (range, 73 to 271) after the beginning of transfusion. In a fifth experiment, represented by the open circles, the pressure was maintained at normal levels for more than 3 hours after transfusion. To provide the best possible chance for survival, the incision in the groin was closed aseptically, and the animal was returned to its cage. It died during the night, however. The sixth animal (solid triangles) died during the 30 mm. Hg period, as mentioned above.

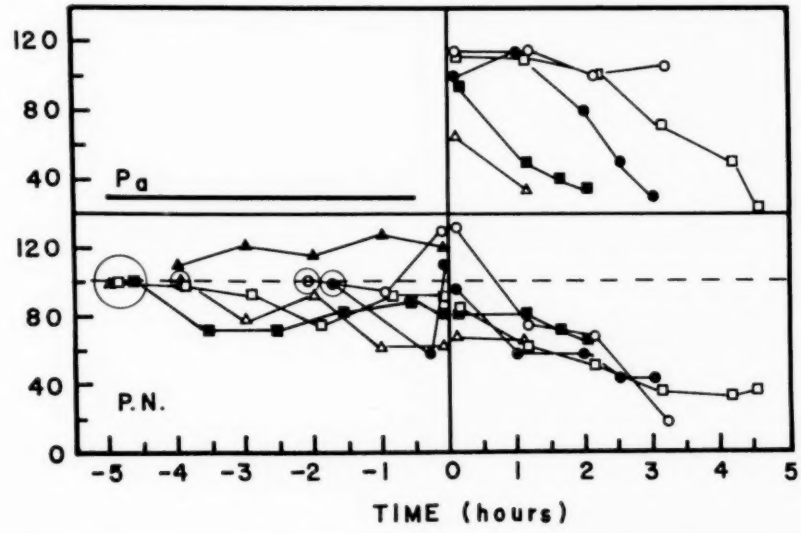

Fig. 1.—The changes in mean arterial pressure *(Pa)* in mm. Hg, and phage neutralization titer *(P.N.)* in per cent of the control value, during hemorrhagic shock. Each experiment is represented by a distinctive symbol. Zero time (represented by the vertical line in the center of the graph) is taken as the time of transfusion of the remaining reservoir blood. The encircled points indicate the time of inception of hemorrhage in each experiment.

The phage neutralization data are presented in the lower half of Fig. 1. For comparative purposes, all values are expressed in terms of per cent of control values. The average control level was 26.0 ± 11.3 units per milliliter. Control samples were drawn in duplicate, 10 minutes apart, and coded. The excellent reproducibility of this assay is affirmed by the fact that in 3 experiments, duplicate control values were identical; in 2, they differed by only 1 unit; and in the remaining experiment, they differed by only 2 units.

During the hypotensive period, some random variations occurred in the phage neutralization titer; but, in general, there was no significant directional change during this period. The titer averaged 82 per cent of control (21.3 ± 5.8 units) 1 hour after the inception of hemorrhage. At the end of the hypotensive period, the mean titer was virtually identical with the control (25.7 ±

12.4). Approximately 7 minutes after reinfusion of the remaining blood, no appreciable alteration was evident in the phage neutralization level (25.0 ± 11.2). However, the titer diminished progressively during the posttransfusion period. The level in the samples drawn just prior to death was 13.6 ± 8.6 units. Even in the experiment in which the animal appeared to be recovering (open circles), the titer dropped precipitously from a posttransfusion level of 131 per cent of control to a preterminal value of 19 per cent 187 minutes later. Despite a blood pressure of 105 mm. Hg at this time, the animal failed to survive through the night after the experiment.

In the two control experiments, the phage neutralization titer did not vary by more than 1 unit during the 7.5 hours of observation.

Aliquots from each blood sample were also used to measure the serum complement level. Similar to the findings of Frank and associates,[3] the complement titer remained quite constant in most animals. In the experiment which terminated prior to reinfusion, no appreciable changes in complement levels were observed. In the 5 remaining experiments, the mean control titer was 27.0 ± 7.2 units per milliliter. The mean preterminal level was 21 per cent lower (21.4 ± 5.0 units per milliliter). However, this decline occurred within the first hour of hemorrhage (21.4 ± 5.1) and it remained constant throughout the remainder of the experiment.

DISCUSSION

The diminution in the phage neutralization titer observed in these experiments is an additional indication of the impairment of the immunologic defense mechanism in hemorrhagic shock.[1] Since phage neutralization is dependent upon the properdin system, the results of these experiments may be interpreted in terms of the reduction of properdin titer which was previously observed by Frank and associates.[3] The results of the present experiments were similar in most respects. The major difference is the time of occurrence of the reduction of immune substances. The properdin titer diminished during the hypotensive stage in the study performed by Frank and collaborators. In the present series, however, the phage neutralization titer remained constant during this interval, and declined in the terminal stages, after transfusion had been completed. At present, it cannot be ascertained whether this discrepancy implies that the zymosan and phage neutralization assays reflect changes in 2 distinct reactions,[4] in 2 related reactions (P_1 and P_2), or that the decline of properdin is simply postponed until after transfusion in the present series for some unknown reason. It is possible that either the 2 assays have different sensitivities to various factors or that the factors themselves are differently depleted by hemorrhagic shock.

SUMMARY

The complement and phage neutralization titers of the serum were measured in dogs subjected to a standard hemorrhagic shock procedure. Complement levels were not appreciably affected. No significant change in the phage neutralization titer was observed during the oligemic phase of shock. During the posttransfusion period, however, the phage neutralization titer declined progressively

and, just prior to death, attained average values of about one half the control levels. These findings provide additional evidence that the immune responses, probably the properdin system, of the organism are adversely affected during shock.

We express our appreciation for the assistance and guidance provided by Drs. Helen Van Vunakis and Lawrence Levine. We would also like to thank Mr. Robert Delaney for performing the complement assays.

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EFFECTS OF CARRIER DIODRAST ON EXCRETION OF I^{131}-LABELED DIODRAST

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After the administration of I^{131}-labeled Diodrast† (iodopyracet), there was significant hepatic uptake and retention of the labeled material which interfered with the evaluation of function of the two kidneys by an external monitoring technique. The prior administration of carrier, or unlabeled, Diodrast (1.0 Gm.) eliminated hepatic uptake of I^{131}-labeled Diodrast and increased its renal excretion approximately twofold. Curves of renal uptake and excretion obtained by external monitoring provided indices of renal function which were not dependent on accurate urine collections.

THERE have been several recent reports of the use of I^{131}-labeled Diodrast with external monitoring over the renal areas for clinical studies of renal function.[2-5] It has been noted that the liver, in addition to the kidneys, takes up and excretes I^{131}-labeled Diodrast; for this reason the radiation detectors have been positioned to avoid the hepatic area.[6, 7]

The present study is concerned with the effects of added stable or carrier Diodrast on the renal and hepatic uptake and excretion of I^{131}-labeled Diodrast and evaluates the use of carrier Diodrast in conjunction with I^{131}-labeled Diodrast in measuring renal function by an external monitoring technique.

MATERIAL AND METHODS

Forty studies of I^{131}-labeled Diodrast excretion were performed on 19 young men with no history of cardiovascular or renal disease. Studies usually extended over 1 half-hour period. Additional observations pertinent to the present study, made in patients with essential hypertension, are also included in this report; the clinical data for these patients are published elsewhere.[8]

The usual dose of I^{131}-labeled Diodrast was 20 μc, which contained less than 1.0 μg of chemical Diodrast. When carrier Diodrast was used, the usual dose was 1 Gm. All doses were given as single intravenous injections. Urine samples were obtained by having the patient void; the plasma samples were obtained by venipuncture using heparinized syringes.

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For external monitoring of renal radioactivity, two collimated NaI(T1) scintillation detectors were directed at the renal areas normal to the skin surface of seated subjects (Fig. 1). The radioactivity of one renal area, either right or left, was measured by a count rate meter and recorded on one side of a dual linear recorder.* The pulses from both detectors were passed to a ratiometer† and the ratio of the radioactivity of one renal area to the radioactivity of both renal areas was recorded on the other side of the dual linear recorder.

By simultaneously averaging and comparing the counting rates of the separate renal areas, the ratiometer provided a statistically valid comparison of the two counting rates immediately, without the necessity of subsequent comparison of separate recordings. The ratio of one counting rate to the sum of both counting rates was recorded on a scale of 0 to 1; when the counting rates were equal, therefore, the scale reading was 0.5, which indicated that 50 per cent of the total radioactivity present was in each renal area. The ratio of the radioactivities in the two kidneys was expressed as the ratio of the percentage in one kidney to the percentage in the other kidney.

To evaluate the counting characteristics of the renal detectors, a simulated model of the kidneys was constructed with glass flasks.

For external monitoring of hepatic radioactivity, a NaI(T1) scintillation detector with a lead shield, collimated to avoid the gall bladder, was pressed against the anterolateral surface of the right side of the chest of the seated subjects; the pulses passed through a count rate meter and were recorded directly on an Esterline-Angus recorder.[9]

Radioactivity of samples which contained I^{131}-labeled Diodrast was measured in a well-type NaI(T1) scintillation counter. Chemical concentration of I^{131}-labeled Diodrast was calculated from the specific activity of the dose used.

TABLE I

COLUMN 1 I^{131}-LABELED DIODRAST ALONE			COLUMN 2 I^{131}-LABELED DIODRAST AND CARRIER DIODRAST		
SUBJECT	RATIO* AT PEAK UPTAKE	MAXIMUM SUBSEQUENT RATIO*	SUBJECT	RATIO* AT PEAK UPTAKE	MAXIMUM SUBSEQUENT RATIO*
A. D.	54:44	60:40	G. E.	50:50	50:50
C. A.†	60:40	60:40	G. E.	54:46	54:46
K. F.	60:40	64:36	L. L.†	50:50	52:48
S. F.	52:48	60:40	E. B.	50:50	50:50
M. H.	56:44	66:34	E. Ho.	48:52	46:54
D. H.	50:50	60:40	D. H.	50:50	54:46
G. K.	52:48	60:40	E. Ha.	50:50	50:50
M. L.	50:50	64:36	A. J.	52:48	54:46
D. L.	52:48	74:26	N. L.	44:56	48:52
D. L.	50:50	64:36	P. G.	48:52	48:52
J. M.	50:50	54:46	R. N.†	50:50	50:50
S. M.	68:32	72:28	S. G.†	52:48	52:48
J. M.	60:40	60:40	A. B.†	56:44	50:50
J. M.	64:36	64:36	A. G.†	52:48	50:50
C. M.	52:48	60:40	G. J.†	50:50	50:50
L. S.	52:48	72:28	L. S.	48:52	50:50
K. S.	58:42	66:34	K. S.	50:50	54:46
K. S.	50:50	58:42			
K. S.	58:42	68:32			
K. S.	48:52	60:40			
Average	56:44	64:36	Average	50:50	50:50

*Right renal radioactivity (per cent) per left renal radioactivity (per cent).
†Essential hypertension.

*Houston Technical Laboratories, Houston, Texas.
†Baird Atomic Associates, Cambridge, Mass.

RESULTS

Effects of Carrier Diodrast on Renal and Hepatic Uptake of I^{131}-Labeled Diodrast.—After the injection of I^{131}-labeled Diodrast alone, there was a rapid uptake of radioactivity in the renal areas. With subsequent excretion of I^{131}-labeled Diodrast, there was an initial fall-off, followed by a plateauing of the renal activity. In all cases, radioactivity in the right kidney area appeared to be greater than that in the left throughout the period of study (Fig. 2). Such apparent right dominance varied in a ratio (right:left) from 54:46 to 74:26, and averaged 64:36, or an average of 64 per cent of the total renal radioactivity appeared to be in the right kidney area (Table I, Column 1).

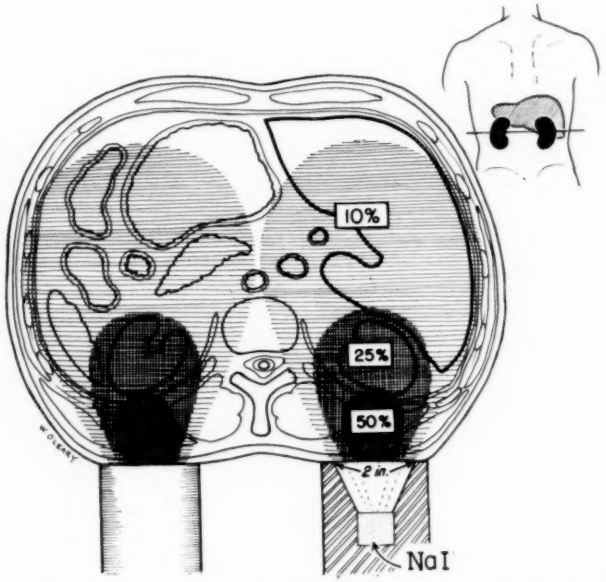

Fig. 1.—Diagrammatic cross section to show liver and kidneys in relation to the monitored areas. Percentages indicate isocount determinations related to counts obtained immediately at the opening of the shield at the periphery of each shaded area.

Monitoring over the liver showed persistent hepatic radioactivity after an initial uptake. This persistence of hepatic radioactivity with decreasing renal radioactivity resulted in an apparently more marked dominance in the right renal area in the ratiometer tracing. After a subsequent injection of stable Diodrast, liver radioactivity decreased, a secondary rise in renal radioactivity was observed, and the apparent right renal dominance disappeared (Fig. 2).

Administration of stable Diodrast prior to the administration of I^{131}-labeled Diodrast diminished hepatic uptake of I^{131}-labeled Diodrast and apparently abolished hepatic retention. Renal uptake of I^{131}-labeled Diodrast was apparently augmented and there was less plateauing in the renal excretory phase. Little shifting of the ratiometer was seen (Fig. 3). When I^{131}-labeled Diodrast was administered after a preliminary liver blocking dose of carrier Diodrast,

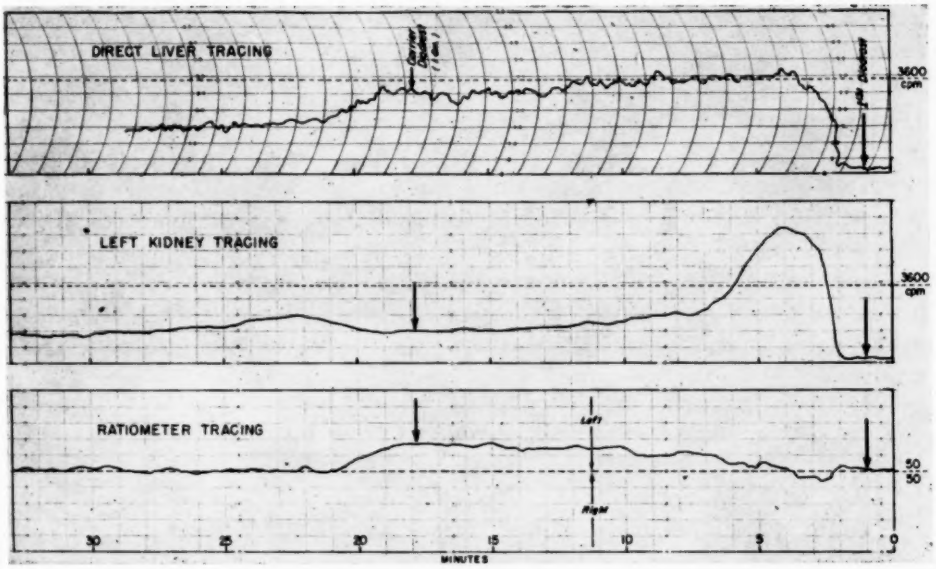

Fig. 2.—Simultaneous monitoring of the kidneys and liver, after administration of I^{131}-labeled Diodrast alone. The liver and kidney tracings are direct recordings of the counting rates in the monitored areas. Where the separate kidney counting rates are equal, the ratiometer tracing is in the midline. In this tracing an upward deflection indicates greater radioactivity in the right renal area than in the left.

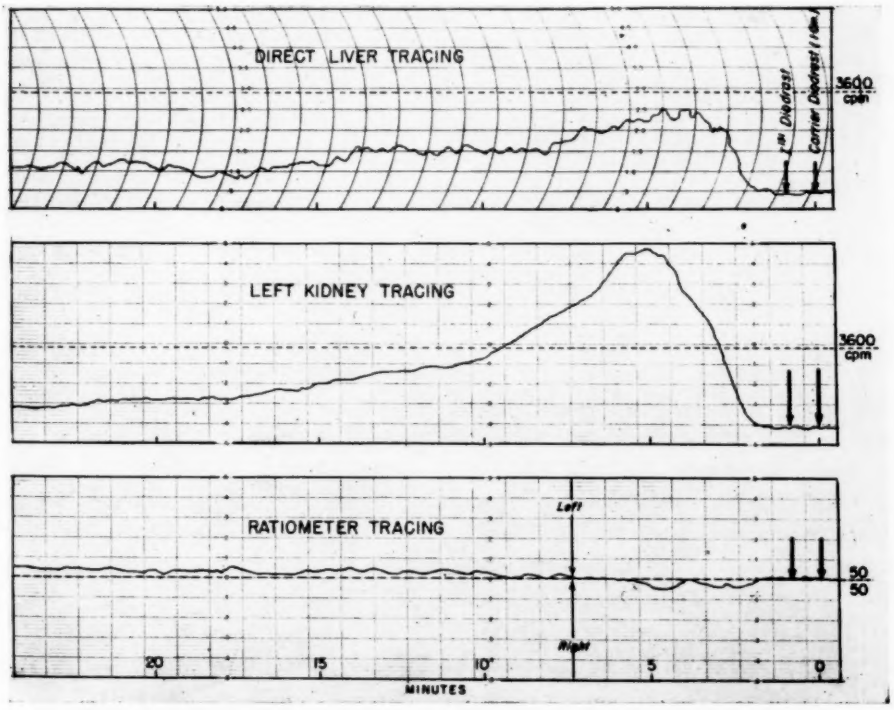

Fig. 3.—Simultaneous monitoring of the kidneys and liver, after I^{131}-labeled Diodrast given after a carrier dose of stable Diodrast. Liver radioactivity is diminished by prior administration of carrier Diodrast, and there is little shift in the ratiometer tracing. Same subject as in Fig. 2.

the ratio of right to left renal radioactivity ranged from 44:56 to 54:46, averaging 50:50 (Table I, Column 2). Several subjects were studied with and without carrier Diodrast to demonstrate these effects in the same individuals (D. H., L. S., and K. S., Table I).

Effects of Carrier Diodrast on Plasma Fall-off of I^{131}-Labeled Diodrast.—With I^{131}-labeled Diodrast alone, there was an initial rapid fall-off of plasma radioactivity, followed within 20 minutes by a plateauing at calculated plasma concentrations of 1.0 μg per cent, temporally related to the plateauing noted in renal radioactivity. When carrier Diodrast was administered during this plateau, a second rise in plasma radioactivity was observed, temporally related to the secondary rise in renal radioactivity and to the decrease in hepatic radioactivity (Fig. 4).

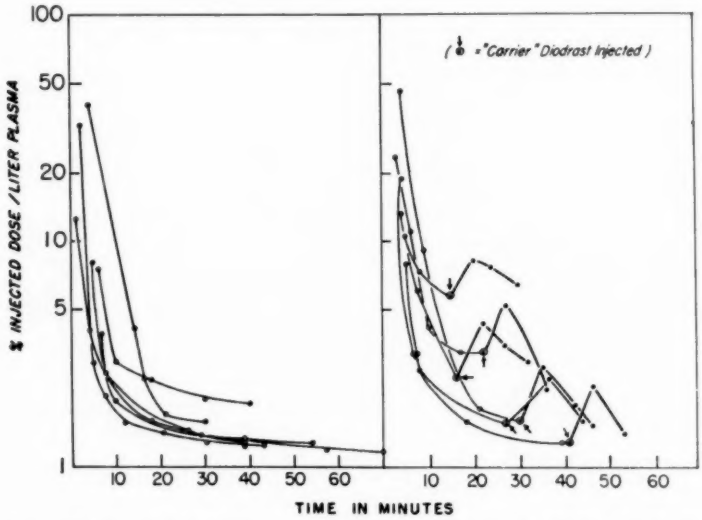

Fig. 4.—Effects of carrier Diodrast on I^{131}-labeled Diodrast plasma disappearance. The increase in plasma I^{131}-labeled Diodrast after administration of carrier Diodrast coincided with the secondary increase in renal radioactivity and decrease in liver radioactivity (cf. Fig. 3).

Effects of Carrier Diodrast on the Urine Excretion of I^{131} Labeled Diodrast.—The 30 minute excretion of I^{131}-labeled Diodrast, administered alone as a single dose to well-hydrated subjects, ranged from 25 to 60 per cent, averaging 41.6 per cent of the injected dose. In dehydrated subjects, the excretion ranged from 34 to 43 per cent, averaging 37 per cent. When carrier Diodrast was administered prior to I^{131}-labeled Diodrast, the 30 minute urine excretion in well-hydrated subjects ranged from 53 to 90 per cent, averaging 71.5 per cent. In similar dehydrated subjects, the excretion ranged from 49 to 71 per cent and averaged 64 per cent (Fig. 5).

The Influence of Background Radioactivity on Renal Monitoring.—Approximately 20 minutes after the injection of I^{131}-labeled Diodrast alone, the plateauing of radioactivity in the renal area included a significant degree of tissue

TABLE II

SUBJECT	HYDRATED		DEHYDRATED		PITRESSIN	
	T½* UPTAKE	T½ EXCRETION	T½ UPTAKE	T½ EXCRETION	T½ UPTAKE	T½ EXCRETION
Bab					67	210
2 hours later					65	230
1 day later			65	235		
4 days later	63	190				
Kim	59	200				
3 days later			61	420		
1 hour later			60	480		
3 months later	60	112				
1 hour later	60	107				
Lau	60	190				
1 hour later	61	180				
LaB	70	190				
1 hour later	61	180				
Fis	61	185				
1 month later			61	282		
Cas	78	210				
3 days later			80	250		
Zach	62	140				
3 days later			62	185		

*Half-life.

background radioactivity, as shown by the plasma sampling. To evaluate this background radioactivity, kidney models were constructed of two glass flasks which contained unequal concentrations of I^{131}. When these were placed in

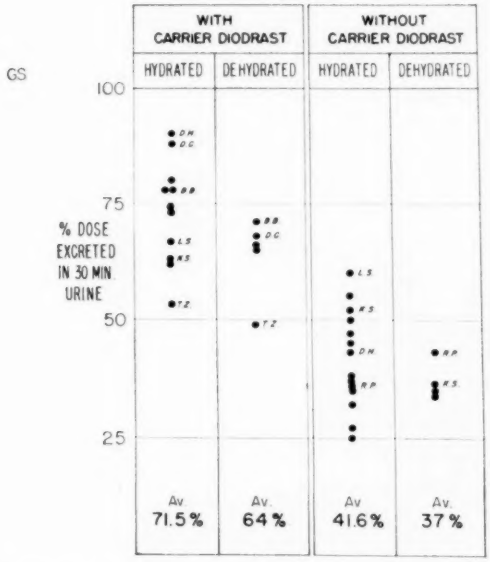

Fig. 5.—Effects of carrier Diodrast on I^{131}-labeled Diodrast excretion. Several subjects (initials) were studied under different conditions. Dehydration had little effect on the 30 minute urine excretion of I^{131}-labeled Diodrast compared with the effect of carrier Diodrast.

front of the detectors, a ratio of 80:20 was obtained (Fig. 6). The subsequent addition of a third container of I^{131}, "seen" by both detectors equally to simulate tissue background radioactivity, decreased the difference between the "kidneys" to a ratio of 68:32. When the kidney models were permitted to empty at equal rates, thereby decreasing their radioactivities relative to the tissue background radioactivity, observed differences between kidneys became even less (Fig. 6).

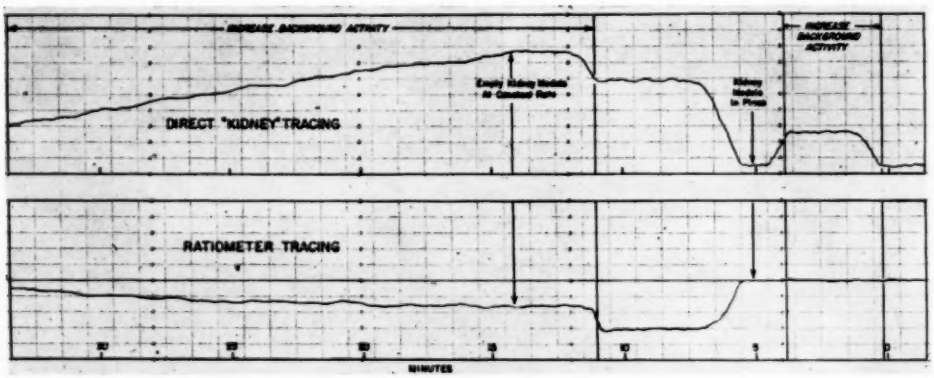

Fig. 6.—The influence of tissue background on differences in renal radioactivity demonstrated by kidney models. The two "kidneys" contained unequal amounts of I^{131} throughout, with no alteration in the relative amounts of I^{131} contained in the models. A high background radioactivity diminished the relative differences between the "kidneys." This became increasingly evident as "kidney" radioactivity fell.

The Rate of Renal Uptake of I^{131}-Labeled Diodrast With Carrier Diodrast.—After the administration of I^{131}-labeled Diodrast, immediately after carrier Diodrast, renal radioactivity in the excretion phase could be plotted on semilogarithmic paper to obtain a rate of excretion (half-life excretion). This slope of excretion could be extended to the ordinate and the difference between this slope and the uptake curve could be plotted to derive a rate of uptake (half-life uptake; Fig. 7). Half-life uptake was quite reproducible (Table II).

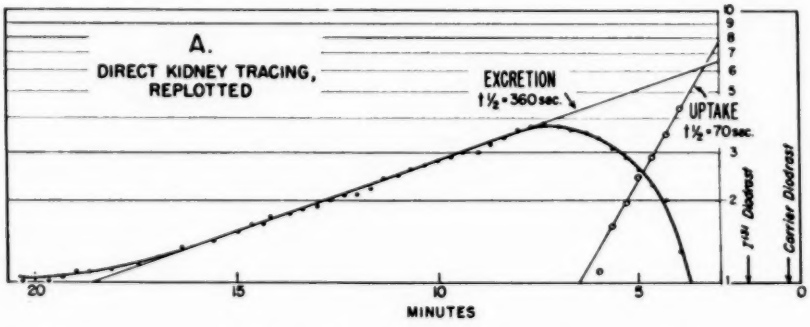

Fig. 7.—Determination of half-life uptake and half-life excretion. An exponential slope for excretion of I^{131}-labeled Diodrast after administration of carrier Diodrast could be plotted on semilogarithmic paper. This slope could be used to derive a rate of renal uptake of I^{131}-labeled Diodrast.

In subjects who were studied in both the hydrated and dehydrated state, a low urine flow prolonged half-life excretion. In one subject, Pitressin did not alter half-life uptake, although half-life excretion was increased.

TABLE III

SUBJECT	T½ UPTAKE (SEC.)	T½ EXCRETION (SEC.)
R. T., at rest	45	185
painful stimulus	47	260
P. W., at rest	47	190
painful stimulus	73	380
J. B., at rest	67	170
painful stimulus	100	480
J.C., at rest	87	380
painful stimulus	100	430
D. C., at rest	80	250
painful stimulus	180	340

Several anxious patients reacted strongly to the stimulus of venipuncture or the test situation. Rates of uptake and excretion of I^{131}-labeled Diodrast under such conditions were prolonged when compared with the results of tests in the same patients when they were not subject to stress (Table III). The influence of these factors is illustrated in the direct tracings recorded at rest and during a mild syncopal attack in the same subject (Fig. 8).

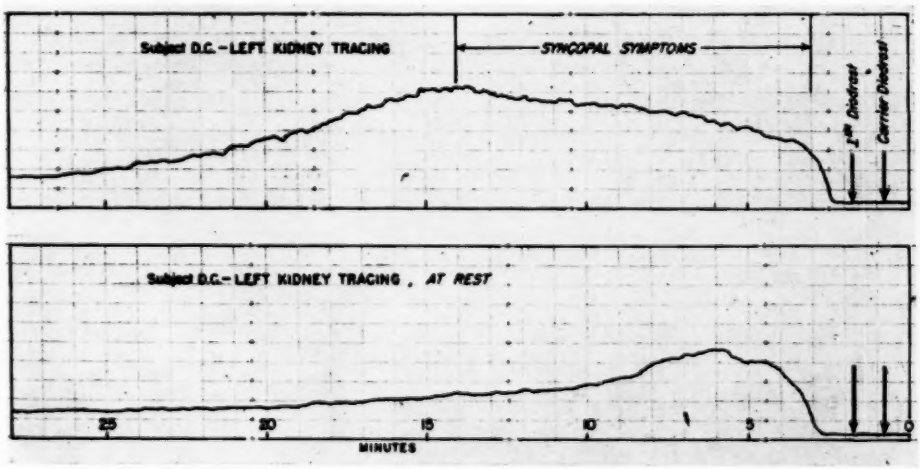

Fig. 8.—Effect of syncope on I^{131}-labeled Diodrast tracings. Continued renal uptake of I^{131}-labeled Diodrast with little excretion was seen during syncope compared with the direct kidney tracings obtained at rest in the same subject.

DISCUSSION

Although the kidneys rapidly excreted a major portion of I^{131}-labeled Diodrast given alone, the liver also took up and retained a significant amount.

The effect of liver radioactivity on the recording of renal radioactivity was greater on the right than the left to a quantitatively unpredictable degree, consistent with the anatomic asymmetry of the liver and its more intimate relationship with the right kidney, which prevented accurate evaluation of the separate kidneys when I^{131}-labeled Diodrast alone was used. Preliminary observations indicated that hepatic uptake of I^{131}-labeled Diodrast occurs at specific binding sites, inasmuch as a variety of organic iodides and substances transported by the liver do not flush I^{131}-labeled Diodrast from the liver as does Diodrast.[7] It was also apparent from common bile duct collections, as well as external monitoring data, that the rate of hepatic excretion of I^{131}-labeled Diodrast is considerably slower than that of the excretion by normal kidneys. The presence of specific binding sites and the slow rate of excretion apparently make it possible to saturate the hepatic transport system for I^{131}-labeled Diodrast with a 1.0 Gm. dose of carrier Diodrast and thus to prevent significant hepatic uptake of I^{131}-labeled Diodrast.

Carrier Diodrast in doses of 1.0 Gm. almost doubled the 30 minute excretion of I^{131}-labeled Diodrast, not only because of blocking of hepatic uptake of I^{131}-labeled Diodrast, but also because of saturation of plasma protein and possibly other peripheral binding sites.[10, 11] This is in contrast to a previous study in which carrier Diodrast decreased the excretion of I^{131}-labeled Diodrast, presumably because doses which exceeded tubular maximum for Diodrast were used.[6]

The effect of carrier Diodrast in increasing the urinary excretion of I^{131}-labeled Diodrast becomes of practical importance if external monitoring techniques are used, in that it results in a decrease in tissue background radioactivity relative to renal radioactivity. Significant functional differences between the kidneys may become evident during the excretion phase[8] and the experiments with the model kidneys would indicate that a high background may obscure such differences.

Derived values for half-life uptake of I^{131}-labeled Diodrast given with carrier Diodrast were reproducible in the same subject and were independent of urine flow. Such measurements may provide information comparable to conventional clearances and be of particular value when variations in urine flow or difficulties in urine collections limit the accuracy of clearance techniques. Factors which alter urine flow, however, influence calculated rates of half-life excretion, which is of particular interest in patients studied for unilateral renal disease.[8] As might be expected, emotional or other stimuli which influence renal hemodynamics may affect the results of I^{131}-labeled Diodrast studies.

SUMMARY

1. Hepatic uptake and retention of I^{131}-labeled Diodrast is obviated by carrier Diodrast.

2. Renal excretion of I^{131}-labeled Diodrast is increased by carrier Diodrast

3. Calculated rates of renal uptake and excretion of I^{131}-labeled Diodrast given with carrier Diodrast offer useful indices of renal function which do not require accurate urine collections.

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STUDIES ON OSMOTIC FRAGILITY OF HUMAN BLOOD PLATELETS

III. Normal and Pathologic Values

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The osmotic fragility of human platelets in hypotonic sodium chloride solutions was studied on the basis of morphologic change and release of the enzyme pyrophosphatase. Ranges of values were obtained on 50 platelet samples from normal individuals between 5 and 60 years of age. By this test, platelets from patients with idiopathic thrombocytopenic purpura manifested increased fragility; those from patients with thrombopathy showed decreased fragility.
—Ed.

IN TWO recent articles[1, 2] we reported on the osmotic fragility of thrombocytes and described the changes in their morphologic appearance in various hypotonic sodium chloride solutions, as observed through the phase contrast microscope and the electron microscope. The two main morphologic changes were the platelets with the swordlike protrusions and the platelet ghosts.

In the present study it has been attempted to establish the normal range of changes in the morphologic appearance of the platelets and the release of enzymes into the different hypotonic sodium chloride solutions. Furthermore, this has been applied as a test to study the thrombocytes of patients suffering from various hemorrhagic disorders, in order to investigate whether these platelets differ from platelets of healthy donors in their osmotic fragility.

MATERIALS AND METHODS

All glassware was siliconized before use. A solution composed of 1 per cent Na_2 Sequestrene (EDTA, ethylenediaminetetraacetic acid) and 1 per cent Triton WR-1339 (oxyethylated tertiary octyl-phenol-formaldehyde polymer) was prepared in 0.7 per cent sodium chloride solution. One milliliter of this solution was transferred into the syringe by means of which 9 ml. blood was subsequently withdrawn. Care was taken to keep the ratio of anticoagulant to blood exactly 1:9.

Preparation of the Platelet Suspension.—The blood was centrifuged immediately after its withdrawal in a 50 ml. round bottomed tube for 7 minutes at 800 r.p.m. (150 g) in an International refrigerated centrifuge with a horizontal head (model PR 2), at a temperature of 5° C. The supernatant plasma containing the thrombocytes was then recentrifuged for 10 minutes at 2,000 r.p.m. (800 g); 0.45 ml. of cold normal saline was added to the platelet sediment and the tube was gently shaken. Under these conditions every 1 c.mm. contained about 2.5×10^6 platelets. No buffer was added to the saline and the pH was 6.5 to 7.0. When more blood was used, 0.05 ml. of saline for each 1 ml. of the blood was added to the platelet sediment. All operations were carried out at a temperature of 5° C. The tube was then put into a refrigerator for 15 minutes and was shaken every 5 minutes until a homogenous concentrate of platelets was obtained.

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The Osmotic Fragility Test.—Standard saline solutions at concentrations of 0.85, 0.44, 0.34, 0.30, 0.24, and 0.1 per cent and thrice distilled water were used in all the experiments.

To each saline solution 0.05 ml. of the platelet suspension was added by means of a siliconized pipette. The tubes were then shaken gently by hand to insure a homogenous distribution of the platelets and were left at room temperature, about 25° C., for 2 hours. The tubes were again shaken gently and 1 drop was transferred to a slide with a siliconized pipette and covered with a cover glass. The preparation was examined immediately under a phase contrast microscope at a magnification of 500 (dry power); the percentage of platelets with a swordlike process and of platelet ghosts was estimated in every saline solution.

Two types of platelet ghosts appeared in all hypotonic NaCl solutions: (1) an empty, bright, very much enlarged envelope of the platelet containing some granules and (2) a swollen rounded platelet of grayish appearance. No distinction in counting was made between these 2 types, and in 0.1 per cent NaCl solution both together constituted 100 per cent of the platelets. The pyrophosphatase released by the platelets in 0.1 per cent NaCl was arbitrarily taken to be 100 per cent.

Estimation of Pyrophosphatase[3].—After the phase contrast examination of the platelet suspensions in the different hypotonic saline solutions, the tubes were centrifuged at 3,500 r.p.m. (2,250 g) for 10 minutes and the supernatants were removed. The supernatant liquid from each tube was added to a solution consisting of 1 ml. Veronal buffer (0.2M), pH 7.2, 1 ml. magnesium chloride (0.1M), and 0.5 ml. sodium pyrophosphate (0.005M). The mixtures, about 3.5 ml. each, were then incubated for 4 hours in a 37.5° C. water bath. The reaction was stopped by the addition of 2 ml. of 10 per cent trichloracetic acid to each tube. The tubes were again centrifuged at 3,500 r.p.m. (2,250 g) for 10 minutes and the supernatants were examined for phosphate by the method of Fiske and Subbarow[4] using a Klett photocolorimeter and a 660 mμ filter. The results were expressed as percentage of the reading obtained with the 0.1 per cent NaCl solution. The latter was taken as standard for 100 per cent pyrophosphatase activity. The reading in thrice distilled water was always higher than the reading at 0.1 per cent NaCl because of the complete disruption of the platelet ghosts in H_2O.

The methods used were as follows: thrombocyte count, method of Brecher and Cronkite[5]; clotting time, method of Lee and White, modified[6]; bleeding time, method of Duke[6]; capillary fragility, method of Hess[7]; clot retraction, method of Macfarlane[6]; and prothrombin consumption, method described by Dacie.[6]

EXPERIMENTAL AND RESULTS

Normal Values.—Fifty platelet samples obtained from individuals between 15 and 60 years of age, known to be suffering from no hematologic affection, were examined. The number of male and female donors was about equal. In 0.85 per cent NaCl the platelets were fairly uniform in size and no ghosts or platelets with swordlike processes were encountered.

At a concentration of 0.44 per cent NaCl the first platelets with swordlike processes were observed amounting to about 5 per cent of the total platelet number (range 0 to 13 per cent with a mean of 4.75 per cent). In this solution the first ghosts also appeared, about 4 per cent (range 1 to 11 per cent with a mean of 3.6 per cent). The pyrophosphatase released by the platelets (expressed as percentage of the amount found in the 0.1 per cent NaCl solution) was 1.5 per cent (range from 0 to 3.6 per cent with a mean of 1.5 per cent).

In the 0.34 per cent NaCl solution, the number of platelets with a swordlike process increased to about 69 per cent of the total number of platelets (range 34 to 90 per cent with a mean of 68.8 per cent). The number of ghosts

also increased in this solution to about 13 per cent of all the platelets (range 4 to 30 per cent with a mean of 13 per cent). The percentage of pyrophosphatase released was 7.5 (range 2 to 14 per cent with a mean of 7.5 per cent).

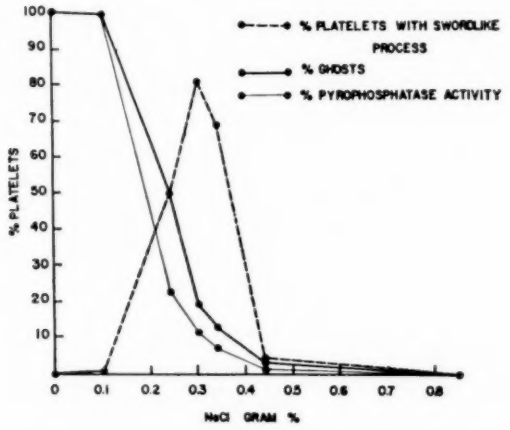

Fig. 1.—Mean values for platelets with swordlike processes, platelet ghosts, and pyrophosphatase activity.

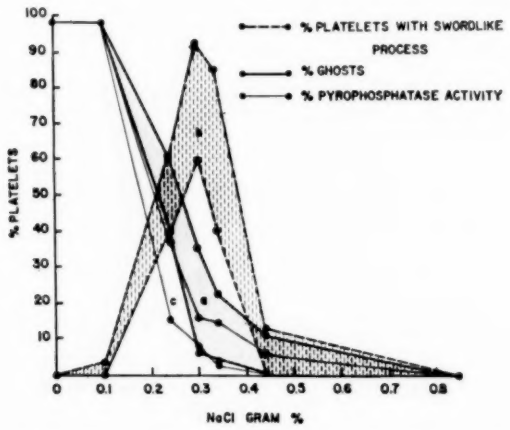

Fig. 2.—Normal range of variations of the swordlike platelets, platelet ghosts, and pyrophosphatase in each NaCl solution.

In the 0.30 per cent NaCl solution there was a further increase both in the platelets with a swordlike process and the ghosts which amounted to 80 per cent of the total platelets (range 51 to 95 per cent with a mean of 80.4 per cent) and 19 per cent (range 4 to 36 per cent with a mean of 19.1 per cent), respectively. The pyrophosphatase activity in this solution was 11.6 per cent (range 2 to 14.7 per cent with a mean of 11.6 per cent).

At a concentration of 0.24 per cent NaCl the number of platelets with a swordlike process markedly decreased, constituting only 50 per cent of the

total platelets (range 40 to 65 per cent with a mean of 50.1 per cent). The number of ghosts in this solution, however, increased further to about one-half the number of the total platelets (range 35 to 60 per cent with a mean of 49.85 per cent). The percentage of pyrophosphatase was 23 (range 13.8 to 36.3 per cent with a mean of 23.1 per cent).

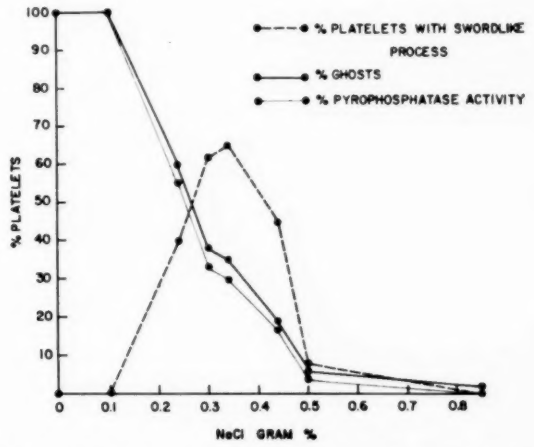

Fig. 3.—Results of the osmotic fragility test of thrombocytes in a case of chronic idiopathic thrombocytopenic purpura.

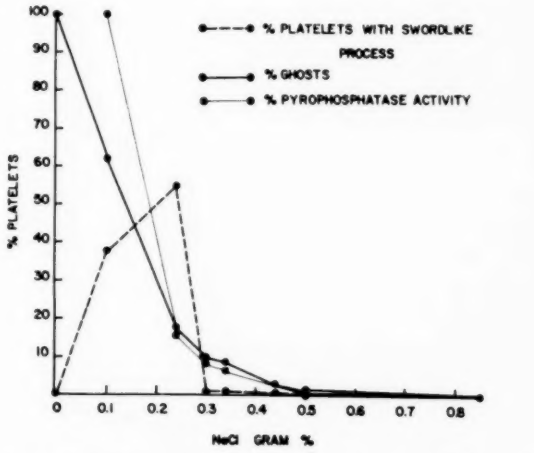

Fig. 4.—Results of the osmotic fragility test of thrombocytes in a case of thrombopathy.

At a NaCl solution of 0.1 per cent, practically no more platelets with sword-like processes were to be seen (range 0 to 6 per cent with a mean of 0.75 per cent). The number of ghosts increased further, approaching 100 per cent, so that nearly all the platelets at this solution had turned to ghosts (range 94 to 100 per cent with a mean of 99.3 per cent). Pyrophosphatase activity at this concentration was arbitrarily taken as 100 per cent.

Each point in Fig. 1 represents the percentage of platelets with swordlike processes, platelet ghosts, and pyrophosphatase activity in the different hypotonic saline solutions. The mean values obtained from all platelet samples were plotted against the NaCl concentration.

The calculation of the normal range in Fig. 2 is based on the assumption that the mean ($\bar{x}$) values for every solution ± 2 S.D. constitute the average range of these values for a normal population.

In Table I the values $\bar{x}$ ± 2 S.D. are calculated for the platelets with swordlike processes. Table II gives these values for platelet ghosts and Table III for pyrophosphatase activity.

TABLE I. MEAN VALUES OF PLATELETS WITH SWORDLIKE PROCESSES AND NORMAL RANGE OF VARIATIONS OF THESE VALUES

NaCl CONCENTRATION	0.85%	0.44%	0.34%	0.30%	0.24%	0.10%	0
$\bar{x}$	0	4.75	68.8	80.4	50.1	0.7	0
$\bar{x}$ ± 2 S.D.	0	0-12.8	37.3-100	61.2-100	39-61.2	0-4.5	0

TABLE II. MEAN VALUES OF PLATELET GHOSTS AND NORMAL RANGE OF VARIATIONS OF THESE VALUES

NaCl CONCENTRATION	0.85%	0.44%	0.34%	0.30%	0.24%	0.10%	0
$\bar{x}$	0.18	3.6	13	19.1	49.85	99.3	0
$\bar{x}$ ± 2 S.D.	0-1.2	0-8.6	3.3-22.7	2.2-36	39.3-60.3	96.6-100	100

TABLE III. MEAN VALUES OF PYROPHOSPHATASE ACTIVITY EXPRESSED IN PERCENTAGE OF THE READING AT 0.1 PER CENT NaCl AND NORMAL RANGE OF VARIATIONS OF THESE VALUES

NaCl CONCENTRATION	0.85%	0.44%	0.34%	0.30%	0.24%	0.10%	0
$\bar{x}$	0	1.5	7.5	11.6	23.1	100	217
$\bar{x}$ ± 2 S.D.	0	0-4.7	1.4-13.6	4.7-18.5	8.3-37.9	100	

Pathologic Values.—The following is a brief account of the results of the osmotic fragility test on thrombocytes from cases of hemorrhagic disorders which have been examined up to now.

Twelve patients suffering from chronic idiopathic thrombocytopenic purpura have been examined. In all patients an increase in the osmotic fragility of the platelets was observed. The platelets with a swordlike process started to appear at a NaCl concentration of 0.6 per cent (normal 0.44 per cent); the platelet ghosts also appeared at lower NaCl concentrations; and the pyrophosphatase values were increased. Five patients showed a very marked deviation from the physiologic values of the osmotic fragility test of thrombocytes, nearly 50 per cent of their cells carrying a swordlike process at a NaCl concentration of 0.44 per cent (normal 5 per cent); 20 per cent of their cells were ghosts at this same solution (normal 0). The values for pyrophosphatase activity in these patients were also markedly increased. In other patients there was only a slight increase in the osmotic fragility of their platelets.

Fig. 3 illustrates the results of the osmotic fragility test of thrombocytes in a patient with chronic idiopathic thrombocytopenic purpura.

Nine patients suffering from thrombopathy have been examined. Two patients were members of the same family. All of these patients suffered from a hemorrhagic diathesis since childhood. They had a normal thrombocyte count, normal clotting time, and a bleeding time over 25 minutes. All of them had an increased capillary fragility. Six of the patients had a very poor clot retraction (less than 25 per cent). Seven patients had an abnormal prothrombin consumption test; their prothrombin consumption indexes were between 50 and 85 per cent. In 2 patients the prothrombin consumption test gave normal results (0 to 40 per cent).

All the patients suffering from thrombopathy showed a decreased osmotic fragility of their platelets. In some cases the first platelets with swordlike processes started to appear only at 0.30 per cent NaCl (normal 0.44 per cent NaCl). There was also a marked reduction in the percentage of platelet ghosts. The relative values for the pyrophosphatase activity were very low at all NaCl concentrations.

In order to investigate the hereditary character of the disease all the available family members of the patients have been examined. None of the healthy parents or siblings showed any abnormal findings; all of the healthy family members showed a normal osmotic fragility test.

Fig. 4 shows the results of the osmotic fragility test of the thrombocytes in a patient with thrombopathy.

DISCUSSION

Earlier attempts to study the osmotic fragility of human platelets have been undertaken[5-10] but as only indirect methods were used the results differed from one investigator to the other according to the method employed. The osmotic fragility of platelets of some animal species has also been investigated.[3, 11] In the present study the osmotic fragility of human blood platelets in normal individuals has been estimated. The examination of the morphologic appearance of the thrombocytes in hypotonic saline solutions and the estimation of the pyrophosphatase released from the platelets into these solutions were found to serve as reliable methods for this purpose. Other enzyme systems have been investigated during these studies, but none was found to be practical for a test. The estimations of dehydrogenase, acid and alkaline phosphatases, besides being less sensitive, necessitated larger amounts of platelets and were found impractical. The estimation of serotonin[2] was also found to be unsuitable for a routine technique.

Values given for the platelets with swordlike processes, platelet ghosts, and pyrophosphatase activity are representative for a normal population. All values falling outside the range of $\bar{x} \pm 2$ S.D. were considered to be abnormal. There are 2 types of abnormal curves, one in cases of thrombocytopenias exhibiting increased osmotic fragility of thrombocytes deviates to the right, whereas the other, found in cases of thrombopathy accompanied by decreased osmotic fragility of thrombocytes, shows a deviation to the left. In the cases of patients suffering from thrombocytopenia the percentage of platelets with swordlike

processes as well as the number of platelet ghosts were increased in the 0.44 per cent NaCl solution and the highest percentage of platelets with a swordlike process was found in the 0.34 per cent NaCl solution instead of the 0.30 per cent NaCl solution. The relative values obtained for pyrophosphatase were also markedly increased.

In cases of thrombopathy practically no platelets with swordlike processes were encountered at 0.44 per cent NaCl. The highest concentration of this kind of cell was reached only at 0.24 per cent NaCl. The percentage of platelet ghosts was also very much reduced in all hypotonic solutions as well as the relative values for pyrophosphatase activity. The decreased osmotic fragility of thromboycetes encountered in cases of thrombopathy may suggest some structural differences in the platelets themselves. This causes the platelets to be more resistant to disintegration, which in turn might be responsible for the increased bleeding tendency in the patients suffering from this disorder.

SUMMARY

The osmotic fragility of human blood platelets from healthy donors has been studied on the basis of the morphologic changes which the platelets undergo in hypotonic sodium chloride solutions and the release of pyrophosphatase. Values for the osmotic fragility of thrombocytes from normal individuals are given. The osmotic fragility test applied to thrombocytes from patients suffering from idiopathic thrombocytopenic purpura showed an increased fragility. Thrombocytes obtained from patients with thrombopathy showed decreased osmotic fragility.

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RADIOSODIUM CONTAMINATION OF A STANDARD PREPARATION OF REACTOR PRODUCED RADIOPOTASSIUM

Its Nature, Amount, and Significance in Biologic Work

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In experiments contrasting the specific activity of potassium in blood and urine after injection of K^{42}, it was observed by chance that there was a seeming disequilibrium between the two. Careful study by chemical methods and by pulse height analysis showed that this seeming disequilibrium was an artefact caused by the presence of trace amounts of sodium in the radioactive potassium. This trace contamination is of considerable significance in biologic work because of the tendency of cells to separate sodium from potassium. In tracer experiments on cell permeability or metabolism, errors of interpretation as great as 30 per cent would arise from this trace contamination. Methods for its detection and freeing the material from the contaminant are discussed.

THE ESTIMATION of exchangeable potassium (K_e) is usually derived from the mean specific activity (counts per minute per milliequivalent) obtained in multiple samples of urine collected 20 to 24 hours after administration of radiopotassium (K^{42}).[1] Some evidence exists[1, 2] that variation in specific activity between successive spot urine samples is greater than can be accounted for by the sum of the known method errors involved. A further investigation was therefore instituted in order to examine the possible errors of the K_e method, with particular reference to a comparison of specific activity in multiple specimens of both urine and plasma. It became apparent in these studies that a systematic difference was present between urine and plasma, and this discrepancy has now been shown to result from the presence of radiosodium (Na^{24}) in the K^{42} used. The purpose of this communication is to examine the effect of this contamination and the possible errors produced in biologic studies, and to indicate the means of minimizing or avoiding them.

METHODS

The K^{42} used in these studies was obtained from Brookhaven National Laboratories as potassium chloride solution, irradiated as potassium carbonate. This target material is identified as "95 per cent pure," with a radio concentration of 1.0 μc per milliliter and a specific activity of 200 μc per gram. Other activity than K^{42} is regarded as negligible.

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A dose of 500 to 800 μc was administered intravenously on 10 occasions in 3 normal female dogs. Twenty to 24 hours later, the animal being unanesthetized and in the fasting state, the bladder was catheterized and emptied, and an indwelling catheter left in situ. Simultaneous samples of venous blood and urine were then collected at approximately 1 hour intervals for 4 to 8 hours.

The radioactivity in each sample of urine and plasma was determined in a well-type scintillation (gamma) counter with a statistical error in counting of less than ± 1 per cent. In a number of experiments, the samples were counted both in the gamma counter and in the thick-walled version of the liquid sample beta counter.[3] Using radio potassium of known purity there is no systematic difference in measured activity as between serum and urine.

Potassium concentration was determined in a Baird flame photometer (lithium internal standard). Repeated measurements of potassium concentration in urine and serum with this instrument in this laboratory agree within ± 1 per cent. The combined reproducibility of flame photometry and counting under these circumstances are in the vicinity of ± 2 per cent.

RESULTS

The results obtained in one experiment are illustrated in Table I, a. A similar pattern of results was obtained in each of 10 experiments, comprising a total of 73 pairs of simultaneously obtained urine and plasma samples. In the

TABLE I

	TIME AFTER K^{42} DOSE (HR.)	SPECIFIC ACTIVITIES		COUNTER
		URINE	PLASMA	
a Untreated samples	24	341	375	Gamma counter
	25	336	370	
	26	351	371	
	27	335	367	
	28	329	359	
	29	336	366	
	Mean	338	368	
	Coefficient of variation	±2.00%	±1.35%	
b Untreated samples	24	223	224	Beta counter
	25	229	222	
	26	229	229	
	Mean	227	225	
	Coefficient of variation	±1.24%	±1.31%	
c Cobaltinitrite treated samples	20	93.5	97	Gamma counter
	21	95.0	94.5	

experiment illustrated, the specific activity of successive spot urine samples agreed within ± 2 per cent (coefficient of variation) and that of successive plasma samples agreed within ± 1.35 per cent of the mean. These limits of variation correspond to the sum of the errors of the methods of counting and flame photometry.

It is however, apparent that a systematic difference is present between the specific activity of urine and that of simultaneously obtained plasma, the value for plasma being consistently higher than that for urine by 8 to 20 per cent throughout the series of experiments.

This discrepancy was significantly greater than the sum of the errors of known method, and was present systematically in each simultaneously obtained pair of urine and plasma samples.

A study of the rate of decay of the original K^{42} solution in the gamma counter showed no deviation from the known half-life of 12.4 hours. However, studies of decay obtained on plasma and urine samples taken after a large intravenous dose of K^{42} revealed a significantly slower rate of decay in the plasma (but not in the urine), the activity after 48 hours being 7 per cent higher than the theoretic figure.

The results obtained when the urine and plasma samples were counted in the liquid sample beta counter are illustrated in Table I, b. The specific activity in urine and plasma was now similar.

A further comparison of specific activity of plasma and urine, in which the gamma counter was used, was made after precipitation of potassium from solution with sodium cobaltinitrite.[4] The potassium precipitate was redissolved and specific activity was determined. The results are illustrated in Table I, c. The difference in specific activity between urine and plasma was completely eliminated by this procedure, irrespective of whether the beta or gamma counter was used.

It appeared, therefore, that the urine-plasma discrepancy might be due to the presence of a contaminating gamma emitting isotope, present predominantly in the plasma.

A pulse-height analysis was carried out in order to confirm this hypothesis and to determine the nature of the contaminant. Gamma ray spectroscopy using a 4 by 4 inch sodium iodide crystal and a 256 channel recorder was performed.* Fig. 1 illustrates the results obtained. In the energy range represented in the figure, the plasma showed two peaks of maximal activity at 1.37 and 1.53 mev., corresponding exactly to the peaks obtained individually with purified solutions of Na^{24} and K^{42}, respectively. In addition, activity was detected at 2.76 mev. both in untreated plasma and in the original K^{42} solution; this energy range is characteristic of Na^{24}. By contrast, the redissolved precipitate† from the cobaltinitrite treated plasma and the untreated urine both showed only a single peak of activity at 1.53 mev.

DISCUSSION

The magnitude of the contamination of the K^{42} with Na^{24} was calculated from (1) the relative decay rates of plasma and standard solution, (2) the relative activities obtained at the energy range 2.76 mev. from the K^{42} and a Na^{24} solution of equal activity (that is, 1 μc), and (3) the difference in plasma specific activity before and after treatment with cobaltinitrite. Each of the three estimates agreed closely with the others which indicates that 0.09 per cent of the K^{42} solution activity was due to Na^{24}. This figure is based on counts done about 72 hours after removal of the target from the reactor. Counts done at 25 hours show approximately 0.06 per cent of activity is due to radiosodium. A later count, for example, a week later, would of course

*Made available through the courtesy of Professor Robley D. Evans of the Massachusetts Institute of Technology. We are grateful to Dr. K. Shivanandan for carrying out this portion of the investigation.

†The potassium cobaltinitrite precipitate is washed in water and redissolved in dilute nitric acid. In preparations containing potassium and sodium with radioactivity present only in the sodium moiety as Na^{24}, this redissolved precipitate is free of radioactivity.

yield a much higher fractional contamination because of the difference in decay rates of the two isotopes. Taking into consideration the relative excitation cross sections of sodium and potassium $\left(\frac{7.7}{1}\right)$ this corresponds to the presence of 0.012 per cent Na^{23} in the target K^{39}, an amount of impurity which is commonly present in analytic grade potassium. Because of the biologic constitution of plasma, where the concentrations of sodium and potassium are 145 mEq. per liter and 4 mEq. per liter, respectively, the percentage activity caused by Na^{24} in plasma is increased 36 times. Because of the relative sensitivity of the scintillation counter for Na^{24} and K^{42} $\left(\frac{5.9}{1}\right)$ the percentage of total counts obtained from Na^{24} becomes 20 per cent.

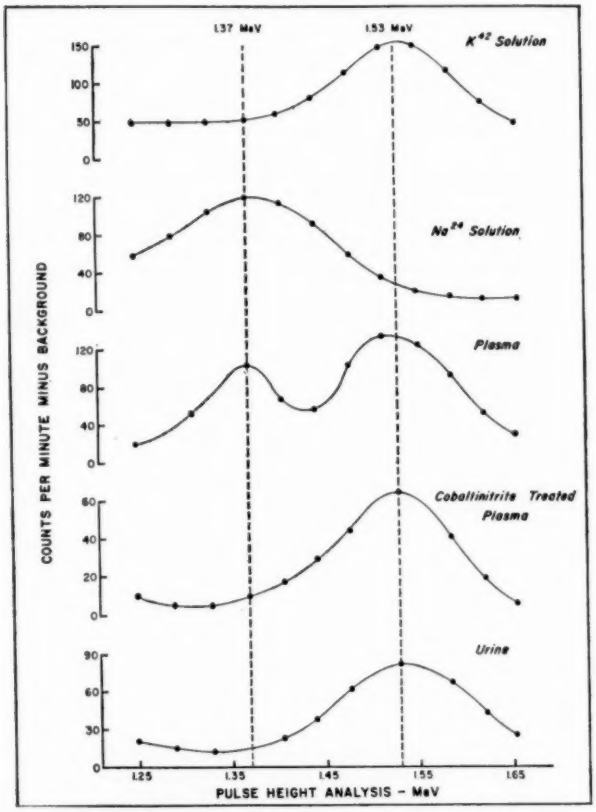

Fig. 1.—Each point on a curve represents the mean of the counts obtained in four successive channels.

Activity of Na^{24} in the original K^{42} solution, although of very small magnitude, was clearly detectable in the energy range 2.76 mev. The main factor, however, which called attention to the contamination was the biologic process by which the sodium : potassium ratio in plasma is maintained at 36 : 1, in contrast to the ratio of from 1 : 1 to 4 : 1 in urine.

It appears, therefore, that estimations of K_e are subject to an error of approximately 20 per cent if untreated plasma samples are counted in a scintillation counter. If urine rather than plasma samples are used, the error caused by radiosodium contamination becomes about 0.5 per cent (when the sodium and potassium concentrations in the urine are equal). If a liquid Geiger counter be used, this error (in urine samples) may be reduced to as low as 0.1 per cent with the thick-walled, and to about 0.3 per cent with the thin-walled variety. With any type of counting equipment, this error is essentially eliminated if chemical separation of potassium in the sample of urine or plasma is performed prior to counting. There are other sources of error in the measurement of the K_e caused by sampling, injecting, syringing, and other handling intrinsic to an isotope dilution technique in man. These figures refer to the error caused by the radiocontaminant.

Chemical separation of radio potassium in serum has been customary in this laboratory by means of cobaltinitrite precipitation for some years past, because our measurements of K_e have been performed as part of a multiple isotope dilution technique.[4-6] Data from such studies are not biased by the radiocontaminant.

Our initial finding, which initiated this investigation (that the untreated specific activities of plasma and urine were seemingly not in equilibrium) raised the possibility that there was some previously unreported "isotope effect" which accounts for disequilibrium of K^{42} in the body pool. The studies reported here indicate that this effect was entirely due to radiocontamination and not to any bizarre lack of isotopic equilibrium.

It may be noted that on no occasion was the variation in specific activity between successive samples of urine greater than the sum of the counting and flame photometry method errors. Our data do not, therefore, support the concept of the existence of biologic variation in specific activity[2] under these experimental circumstances.

Attention is called to the fact that most biologic systems exhibit a fine discrimination between sodium and potassium, and, therefore, that this small fractional contamination of radiopotassium with radiosodium is of unusual significance in biologic work. Experiments on cell or membrane permeability, sodium-potassium exchange, cellular exclusion of sodium, and related matters will suffer a considerable systematic error if this detail of radioisotope contamination is overlooked.

SUMMARY

The K^{42} solution obtained from Brookhaven National Laboratories is shown to be contaminated with Na^{24} to the extent of 0.06 to 0.09 per cent. This may result in an error of 20 per cent in the estimation of plasma potassium specific activity, because of the high sodium : potassium ratio in plasma and the greater sensitivity of the scintillation counter for Na^{24}. The error may be avoided either by counting untreated urine samples in a beta counter, or by chemical separation of the potassium from sodium prior to counting.

The addition of carrier sodium to the target material after irradiation, and the complete separation of potassium by cobaltinitrite precipitation would be an alternative method of purification.

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HISTOPLASMIN SENSITIVITY IN ISRAEL

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Sensitivity tests to histoplasmin in nonendemic areas are of great interest to the epidemiologist and to the clinician for the understanding of pulmonary findings of undefined cause. Isolated cases of histoplasmosis have been reported from Mediterranean countries, but no case of histoplasmosis has yet been detected in Israel whose population originates from almost all continents.

No positive skin reaction to histoplasmin was found in 136 persons originating from European and Western countries and 163 individuals from Asio-African countries. Two hundred and thirty four of 260 of this series tested simultaneously for tuberculin sensitivity revealed positive reactions. Twelve of 30 tested for trichophytin sensitivity had positive reactions. These results indicate that there is no cross sensitivity between histoplasmin, tuberculin, and trichophytin.

PULMONARY calcifications in patients with negative tuberculin and positive histoplasmin skin tests have necessitated the revision of the clinical and epidemiologic concept of histoplasmosis and the interpretation of the pulmonary findings as a specific manifestation of histoplasmosis.[2, 5, 7, 9, 13] Sporadic outbreaks of small epidemics of histoplasmosis have been reported.[3, 7, 10] The clinical picture was that of a rapidly self-healing pneumonitis with positive skin sensitivity to histoplasmin. Histoplasmin sensitivity tests in hitherto nonendemic areas outside the United States of America have shown that histoplasmosis might be far more widely prevalent than clinical awareness or the actual recovery of the parasite from patients, animals, and soil would indicate.[1, 3, 4, 8, 14, 15]

Reactivity to histoplasmin is apparently completely specific. The testing of large groups of people in various parts of the world initiated by the World Health Organization will eventually result in a complete world map of histoplasmin sensitivity. Such sensitivity, detected in areas where no actual cases of histoplasmosis have been found, is of great importance for the interpretation of pulmonary findings of undetermined cause.

The population of Israel consists of persons born in the country and of persons who have immigrated from almost all continents. Although isolated cases of histoplasmosis or histoplasmin sensitivity have been reported from Turkey, Italy, Spain, Portugal, North African countries, and Egypt, no case of histoplasmosis in Israel has yet been detected. The distribution of ethnic groups in the hospital corresponds fairly well with the distribution in the

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general population; therefore, a survey on histoplasmin sensitivity in hospitalized patients can be assumed to give a reasonably accurate estimate of the incidence of histoplasmin reactors among the general population.

The present report is based on tests carried out in 299 patients hospitalized in the departments of chest diseases, internal medicine, and dermatology, and outpatients visiting these departments. The patients (according to their origin) came from European and Western countries (136) and from Asian or African countries (163).

Histoplasmin.—Histoplasmin produced by Eli Lilly & Company designated lot CTS-5 or histoplasmin lot H-42 prepared by the Public Health Service of the United States was used.[12] The commercial vaccine was diluted to 1:100 and 1:1,000 with saline containing 0.5 per cent phenol; the preparations were used as quickly as possible. (The stock vaccine was kept in the refrigerator.) The vaccine from the U. S. Public Health Service was used in its original 1:100 dilution. The control consisted of normal saline solution containing 0.5 per cent of phenol. Intracutaneous injections of 0.1 ml. were made on the inner aspects of the arm or forearm. The syringes and needles were used exclusively for histoplasmin testing. Most of the patients were tested for tuberculin sensitivity and some for trichophytin sensitivity. The results were read after 48 hours according to the criteria used by the World Health Organization.

RESULTS

Three hundred and nineteen patients were tested. Most patients were in the age group of 20 to 60 years, the youngest was 3 years of age and the oldest was 74 years of age. A summary of some clinical data is presented in Table I. Of 262 patients tested simultaneously for tuberculin sensitivity 234 had positive reactions. Of 51 patients tested for trichophytin sensitivity 19 had positive reactions. No positive reaction to histoplasmin occurred in any of the patients.

TABLE I. DISEASES AND ORIGIN OF 319 PATIENTS

ORIGIN	TUBERCULOSIS OF LUNGS	NON-TUBERCULOUS LUNG DISEASES	NON-INFECTIOUS INTERNAL DISEASES	SKIN DISEASES	TOTAL
African and Asian countries	32	94	21	29	176
European and Western countries	40	60	18	25	143
Total	72	154	39	54	319

These results indicate that there is no cross sensitivity between histoplasmin and tuberculin or trichophytin and suggest the specificity of the histoplasmin test.

SUMMARY

No histoplasmin skin sensitivity was found among 319 patients examined in various departments of the Hadassah University Hospital. No cross sensitivity between histoplasmin or tuberculin or trichohytin was found.

Thanks are due to Eli Lilly & Company and to Drs. Palmer and Edwards of the U. S. Public Health Service for the generous supply of histoplasmin.

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INACTIVATION OF HAGEMAN FACTOR BY DIISOPROPYL-FLUOROPHOSPHATE (DFP)

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Previous studies have suggested that Hageman factor is a proteolytic enzyme. To test this hypothesis, the ability of diisopropylfluorophosphate, a highly specific inhibitor of enzymes with esterase properties, to inactivate Hageman factor was investigated. Hageman factor was demonstrated to be inhibited by diisopropylfluorophosphate.—ED.

HAGEMAN factor[1] is ". . . a clot promoting factor present in normal barium sulfate adsorbed heated plasma," which is absent in the plasma of individuals with the so-called Hageman trait. Hageman factor has been shown to be the agent responsible for the activating effect of glass on clotting[2-4] and it has been proposed[3] as the agent responsible for the production of pain producing substance[5] (plasma kinin). Hageman factor is believed to be activated by glass, after which it reacts with a second substance present in normal plasma to yield a clot promoting substance.[3, 6] The finding that activated Hageman factor could be inhibited by soybean trypsin inhibitor suggested the possibility that Hageman factor is a proteolytic enzyme similar in its properties to trypsin. As a critical test of this hypothesis the ability of diisopropylfluorophosphate (DFP)* to inactivate Hageman factor was investigated.

MATERIALS AND METHODS

Crude Hageman factor was prepared from freshly drawn Ba SO_4 adsorbed, heated human plasma by precipitation between 30 to 40 per cent saturation with $(NH_4)_2$ SO_4 and two dialyses against phosphate buffer, pH 5.5, as described by Ratnoff and Rosenblum.[4]

The Hageman factor was assayed by its ability to decrease the clotting time of normal human plasma recalcified in silicone coated test tubes.[4]

When preparations of Hageman obtained in the above manner were incubated at 37° C., activity was found to have increased to a maximum after 1 hour. In all experiments, therefore, the Hageman factor preparation was incubated at 37° for 1 hour before use.

A 0.01M stock solution of DFP in triethanol amine buffered saline (pH 7.3) was made just before the test, and appropriate dilutions were made from this.

Dialyses were performed in Visking casings at 4°.

Hageman factor was added to varying concentrations of DFP. One tenth milliliter samples were removed and assayed for activity immediately after the addition of DFP and after 3 hours incubation at 37°. At the end of this time the mixtures were placed in Cellophane sacs and dialyzed overnight against 0.15M NaCl. They were then tested again for Hageman factor activity.

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RESULTS

Table I demonstrates that incubation with 1×10^{-3}M DFP inactivated Hageman factor activity almost completely and even at 5×10^{-5}M DFP, some inactivation was evident. As would be expected, the higher concentrations of DFP showed a direct inhibiting effect on clotting as seen in the results obtained at zero time. Nevertheless, the inactivation of Hageman factor activity by DFP was still manifest after dialysis to remove DFP.

TABLE I. INHIBITION OF HAGEMAN FACTOR BY DFP

	DFP CONCENTRATION (1×10^{-4}M)					NO HAGEMAN FACTOR
	10	5	1	0.5	0	
Clotting time immediately after addition of DFP (minutes)	17½	13½	11	11	11	23
Clotting time after 3 hour incubation at 37°	26	25	26	23	11¾	26
Clotting time after 18 hour dialysis at 4°	27	23	22	20½	13¾	28

Three tenths milliliter of Hageman factor + DFP + triethanol amine buffered saline (pH 7.3) to make 1 ml. One tenth milliliter samples were removed at indicated times and added to 0.2 ml. of citrated plasma and 0.1 ml. 0.05M $CaCl_2$ in silicone coated tubes. In the tube with no Hageman factor 0.3 ml. of buffered saline was added.

Through the generosity of Dr. Oscar Ratnoff a sample of Hageman deficient plasma from a patient with Hageman trait was obtained. Repetition of these experiments in which the ability of DFP treated crude Hageman factor to reduce the clotting time of Hageman deficient plasma is used gave essentially the same results.* The preparation of untreated Hageman factor reduced the clotting time of the Hageman deficient plasma from greater than two hours to 7½ minutes. Treatment of the preparation of Hageman factor with 5×10^{-5}M DFP yielded a preparation which gave a clotting time of 16 minutes.

A preparation of crude Hageman factor was also prepared from plasma obtained from citrated blood which had been allowed to stand 40 days at 4°. The method of preparation was the same as that used with fresh plasma and a fraction was obtained which had high activity in reducing the clotting time of normal recalcified plasma. Nevertheless, DFP had very much less inactivating on this preparation than on preparations made from fresh plasma.

DISCUSSION

DFP has been shown to be a highly specific inhibitor of enzymes with esterase properties.[7] This not only includes acetylcholine esterases but also proteases with esterase properties such as trypsin and chymotrypsin. Thus the finding that DFP inhibits Hageman factor activity at concentration levels characteristic of its inhibition of these latter enzymes suggests very strongly that Hageman factor is an esterase. Taken in conjunction with its reported inhibition by soybean trypsin inhibitor[2] the results with DFP provide powerful support for the hypothesis that Hageman factor is a proteolytic enzyme. This,

*The results of the DFP inhibition were described to Dr. Oscar Ratnoff who later informed us he was able to confirm them with the use of highly purified Hageman factor.

if fully substantiated by later work, would mean the involvement in the clotting process of at least 2 different enzymes of the same general type, Hageman factor and prothrombin.

SUMMARY

Hageman factor activity has been shown to be inhibited by diisopropylfluorophosphate (DFP). It is suggested that Hageman factor is an esterase similar in nature to trypsin, thrombin, etc.

I wish to thank Dr. Oscar Ratnoff for his generosity in providing me with plasma from a Hageman deficient patient, and for his kindness in informing me of the results of unpublished work.

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FAILURE OF PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY IN THE NEWBORN HUMAN INFANT

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At birth, and for a variable period thereafter, the newborn human has a limited capacity to produce circulating antibody in response to injected antigens. Maturation of the immune response coincides with the development of plasma cells and secondary follicles in the stimulated lymph nodes.

The capacity to express delayed hypersensitivity is also absent at, and shortly after, birth. Clinical and experimental observations are limited to infants unintentionally infected with tubercle bacilli by their mothers and to infants given Bacillus Calmette-Guérin (B.C.G.) vaccination. In these infants, delayed hypersensitivity can occasionally be demonstrated as early as 21 days of age and uniformly by 4 to 6 weeks of age.

It is well known that the unresponsive neonate can accept and utilize passively transferred antibody, but only Schlange's study suggested that the neonate could manifest delayed hypersensitivity to tuberculin after exchange transfusion. Our studies with a variety of passive transfer techniques, indicate that newborn infants, 1.5 hours to 1 month of age, are unable to accept the passive transfer of delayed hypersensitivity.

These observations indicate that newborn infants are defective in their capacity either to express delayed allergy, or to accept passively transferred delayed type hypersensitivity. This deficiency suggests that the recipient of so-called passively transferred hypersensitivity may participate actively in the development of the delayed allergy. In addition, the observation may have important bearing on understanding of the basis for deficient resistance to infection which characterizes the neonate.

THE LIMITATIONS of the newborn infant with respect to the capacity to express an active immune response have been the subject of much recent investigation, which has produced some apparently conflicting data. Most available evidence supports the conclusion that at birth, and for a variable period thereafter, the newborn has a limited capacity to produce circulating antibody in response to injected antigens.[1-3] The morphologic expressions of the mature

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immune response, plasma cell production and secondary follicle formation in the stimulated lymph nodes, are not detected during this period of life, but they appear later in direct relationship to the capacity to produce antibody.[4] In the rabbit and chick a similar neonatal immunologic unresponsiveness has been defined.[5-8]

The studies of newborn rabbits by Sterzl and Holub[9] suggest that the immature rabbit can support antibody production if a deficiency in the inductive phase of the immune response can be overcome. In contrast, the observations of Dixon and Weigle[6, 7] suggest that the unresponsiveness in the newborn rabbit lies in the productive phase of the immune response as a result of an internal environment inadequate to support antibody production. On the other hand, some recent evidence[10, 11] suggests that the newborn human and rabbit do have the capacity to produce antibodies to certain antigens, but that this response differs qualitatively from the mature response and is not associated with the appearance of plasma cells.

In contrast to the number of studies of the induction of the humoral antibody response in the neonatal period, few data are available to indicate the status of the newborn human with respect to the capacity to actively develop delayed allergy. Clinical observations on infants infected with tubercle bacilli early in the neonatal period, and observations from those countries which vaccinate with B.C.G. at birth, are that delayed allergy to tuberculin can rarely be elicited before 3 weeks of age. Furthermore, Waksman and Matoltsy[12] were unable to passively transfer tuberculin delayed reactivity to newborn guinea pigs. In contrast to this, Schlange[13] has reported the passive transfer of delayed allergy to newborn infants by exchange transfusion with blood from tuberculin-positive donors.

These conflicting data and the unsolved question of whether the transfer of delayed allergy in man is a passive process[14] or whether active participation[15] by the recipient plays an important role, led us to investigate this problem. Accordingly, advantage was taken of the various clinical needs of newborn infants to receive transfused blood elements in order to determine their capacity to express delayed hypersensitivity after receipt of living leukocytes from known sensitive donors. Contrary to the data of Schlange, no evidence of passive transfer of delayed hypersensitivity to the newborn infants was observed.

MATERIAL AND METHODS

Procedure for Obtaining Leukocyte Suspensions.—Four hundred to 500 ml. of blood was drawn from the antecubital vein of sensitive donors through a 15 gauge needle and polyethylene tubing into a silicone coated vacuum bottle (Baxter) or a plastic donor bag (Fenwal) which contained heparin (5 mg. per 100 ml. blood) as an anticoagulant. The fluid phase of the blood was then adjusted to a concentration of 1.2 per cent dextran with commercial 4.7 per cent dextran (Baxter). The red blood cells were allowed to sediment in this mixture for 50 minutes at room temperature, after which the supernatant, which contained white cells in suspension, was withdrawn with sterile 50 ml. siliconized volumetric pipettes or plasma transfer sets (Baxter) and transferred to 250 ml. siliconized centrifuge tubes. The suspension was centrifuged at 500 to 1,000 r.p.m. for 20 minutes, after which the supernatant was poured off and the leukocytes which had collected were resuspended

by gentle agitation in 5 to 10 ml. of Hanks buffered saline solution. At this time in one or two of the experiments, the cells were again centrifuged, the supernatant was poured off, and the cells were again resuspended. It was observed that the apparent viability of the cells was reduced by this manipulation, and the cells were not washed except in the earliest experiments. Immediately after resuspension of the cells and while their number and viability were being evaluated from a small aliquot, the cell suspension was injected subcutaneously and/or intramuscularly into the buttocks or shoulder region of the newborn or immunologically mature recipients. The number of cells was evaluated by standard counting procedure, using red blood cell pipettes, and the viability of the cells was estimated by their capacity to resist staining with trypan blue.[16] Sterile siliconized glassware and commercially obtained plastic bags were used throughout these experiments, and all procedures were carried out as rapidly as possible in an effort to maintain viability of the white cells being transferred.

Exchange Transfusion Procedures.—The standard umbilical vein technique used in exchange transfusion of erythroblastotic babies was used throughout. Five hundred ml. of blood from a donor with a known hypersensitivity response was drawn as before from the antecubital vein into a siliconized bottle which contained heparin (5 mg. per 100 ml. blood). The blood was typed and cross matched. Total white cell counts and viability studies were done at the beginning and termination of the transfusion. The evaluation was made in this manner to eliminate error caused by the death of cells or their packing in the bottle during the transfusion. The total number of viable leukocytes was then estimated from the volume of the exchange transfusion and an average of the total number of cells and percentage viability in the initial and terminal evaluations.

Transfer of Viable Peripheral Leukocytes Plus Transfer of Viable Bone Marrow Cells.—Preparation of leukocytes and their transfer were achieved as in the first method described. In addition, bone marrow was aspirated from the donor's sternum and multiple sites on the iliac crests. Between 23 and 50 ml. of bone marrow was aspirated in the individual cases. Viability estimates and total leukocyte counts were performed and the whole bone marrow was injected intramuscularly and subcutaneously into each of 6 to 8 sites in the thighs, buttocks, and arms of the recipients.

Skin Testing.—Each of the donors was skin tested prior to or immediately after cells were obtained for transfer. The recipient infants were skin tested between 24 and 120 hours after transfer and, in some instances, at weekly intervals for several weeks. The newborn babies were not tested prior to transfer. The immunologically mature control children and adult recipients were tested once prior to the transfer of cells and one or more times after transfer.

All skin tests were carried out as follows: 0.1 ml. of skin test antigen was injected intradermally on a smooth skin surface, usually on the forearm or medial aspect of the thigh. The injections were performed between 24 and 120 hours after transfer of cells. Antigens used were old tuberculin 1:1000, histoplasmin 1:100 (Lilly), 10 units SK and 2.5 units SD,* mumps commercial skin test antigen (Lederle), Schick test control (delayed allergy elements of diphtheria bacillus), Candida albicans (1:100), and Trichophyton (1:30) antigen.† The skin tests were evaluated 24, 48, and 72 hours after injection of antigen.

Simultaneous Transfer of Delayed Allergy to Newborn and Immunologically Mature Individuals.—In these experiments, aliquots of donor cells, obtained as described, were injected intramuscularly into the newborn and immunologically mature recipients at the same time, and skin testing was carried out at the same intervals thereafter. The infants received a larger number of cells per unit of weight than did the mature recipients, since no attempt was made to adjust the number to body size.

*Varidase, streptokinase-streptodornase, Lederle Laboratories, Pearl River, N. Y.
†Both prepared by Hollister-Stier Laboratories, Los Angeles, Calif.

TABLE I. ATTEMPTED PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY IN NEWBORN AND IMMUNOLOGICALLY MATURE INDIVIDUALS WITH VIABLE LEUKOCYTES OBTAINED FROM THE PERIPHERAL BLOOD

			SENSITIVITY RESPONSES								PASSIVE TRANSFER RESPONSES			
DONOR	AGE	SEX	OT	SK-SD	MUMPS	DIPHTHERIA TOXOID CONTROL	ESTIMATED NO. OF CELLS TRANSFERRED	% VIABLE	RECIPIENT	AGE	SEX	OT	SK-SD	MUMPS	DIPHTHERIA TOXOID CONTROL
R. G.	35	M	—	4+	3+	4+	1.14×10^9	90	K. P.	8 hr.	F	0	0	0	0
W. W.	29	M	4+	4+	4+	4+	5.4×10^8	99	L. A.	16 hr.	M	0	0	0	0
M. E.	31	M	neg.	3+	3+	1+	2.1×10^9	85	L. E.	1 mo.	F	0	0	0	0
M. G.	35	M	4+	4+	4+	0	1.2×10^9	100	B. G.	6 hr.	F	0	0	0	0
J. S.	—	M	0	0	2+	0	1.1×10^9	70	G. R.	20 hr.	F	0	0	0	0
J. G.	32	M	2+	4+	4+	neg	2.0×10^9	95	J. G.	3 hr.	M	0	0	0	0
R. G.	35	M	—	4+	3+	4+	1.0×10^9	88	C. C.	8 yr.	M	0	2+	1+	2+
S. Z.	26	M	4+	4+	3+	—	1.6×10^9	90	T. R.	11 yr.	M	2+	1+	0	0
B. P.	26	M	2+	4+	3+	—	1.3×10^9	98	A. R.	62 yr.	M	0	2+	1+	0

RESULTS

In Table I are summarized the results of attempts to passively transfer delayed type hypersensitivity from highly sensitized donors with leukocytes separated from the peripheral blood and injected subcutaneously and intramuscularly into newborn infants. Even though the donors were highly sensitive and large numbers of viable cells were obtained for transfer in each instance, no evidence of successful passive transfer of sensitivity to tuberculin, streptococcal products, mumps antigen, or diphtheria toxoid was obtained in the newborn infants studied. When comparable numbers of leukocytes, obtained from similarly sensitive donors, were transferred to nonsensitive but immunologically mature recipients, transfer of delayed allergy was regularly achieved. In additional studies carried out during the same period, attempts to passively transfer delayed allergy with peripheral blood leukocytes from donors who had a high degree of sensitivity to immunologically normal, nonsensitive recipients have regularly been successful. In 14 of 17 such attempts, we have been able to transfer delayed allergy to one or another of the following allergens: 2,4 dinitrofluorobenzene, tuberculin, streptococcal antigens, mumps antigen, Ascaris antigen, Candida or Trichophyton, and cat scratch antigen. Thus, using this technique, newborn infants appear to be refractory to the passive transfer of delayed allergy with leukocytes from the peripheral blood.

Tables II and III contain the results of our attempts to transfer delayed allergy to infants with erythroblastosis fetalis by use of the exchange transfusion method. Each infant included in Table II was given an exchange transfusion for erythroblastosis fetalis. Five hundred to 600 ml. of blood was exchanged via the umbilical vein by standard technique similar to that employed by Schlange.[13] In none of the recipient infants was delayed allergy demonstrated, even though each donor had a high degree of delayed allergy to one or more of the antigens studied at the time the exchange was carried out. Similarly, those infants included in Table III were given exchange transfusions for erythroblastosis fetalis. In addition, leukocytes separated from an additional 100 to 200 ml. of fresh blood from the same donor were injected subcutaneously or intramuscularly. Again, none of these infants accepted the transfer of delayed allergy.

In Table IV are summarized three experiments in which transfer of delayed allergy to human newborns was attempted, using not only large numbers of peripheral leukocytes but also an appreciable number of cells obtained from either single or multiple bone marrow aspirations. No evidence of transfer was obtained.

Finally, three critical transfer studies are summarized in Table V. In each of these experiments, peripheral leukocytes were obtained from highly sensitized donors. The viable leukocytes suspended in saline solution were divided into aliquots which were injected subcutaneously into a newborn infant and into a nonsensitive, immunologically mature recipient. The results were clear-cut. The cells transferred to the newborn infant failed to induce the development of

TABLE II. ATTEMPTED PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY IN NEWBORN INFANTS WITH VIABLE CIRCULATING LEUKOCYTES DURING EXCHANGE TRANSFUSION FOR ERYTHROBLASTOSIS FETALIS

			SENSITIVITY RESPONSES									PASSIVE TRANSFER RESPONSES			
DONOR	AGE	SEX	OT	SK-SD	MUMPS	DIPHTHERIA TOXOID CONTROL	ESTIMATED NO. OF CELLS TRANSFERRED	% VIABLE	RECIPIENT	AGE	SEX	OT	SK-SD	MUMPS	DIPHTHERIA TOXOID CONTROL
D. B.	24	F	4+	4+	3+	—	1.5×10^9	75	G. R.	20 hr.	F	0	0	0	0
T. B.	21	M	0	4+	3+	1+	3.3×10^9	95	B. M.	3 days	M	0	0	0	0
C. H.	27	F	0	2+	0	0	5×10^9	95	Y. E.	8 hr.	M	0	0	0	0
B. S.	26	M	0	4+	4+	0	4.5×10^9	95	B. K.	16 hr.	M	0	0	0	0

TABLE III. ATTEMPTED PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY IN NEWBORN INFANTS WITH LEUKOCYTES INJECTED SUBCUTANEOUSLY AND INTRAMUSCULARLY AND WITH LEUKOCYTES INJECTED INTRAVENOUSLY DURING EXCHANGE TRANSFUSION

			SENSITIVITY RESPONSES				ESTIMATED NO. OF CELLS TRANSFERRED	% VIABLE	RECIPIENT	AGE	SEX	PASSIVE TRANSFER RESPONSES			
DONOR	AGE	SEX	OT	SK-SD	MUMPS	CANDIDA						OT	SK-SD	MUMPS	CANDIDA
W. D.	25	M	0	3+	4+	2+	3.5×10^9*	70	B. T.	8 hr.	M	0	0	0	0
							7.4×10^8†	70							
C. C.	27	M	0	4+	1+	3+	2.7×10^9*	80	B. T.	3 days	M	0	0	0	0
							4.7×10^8‡	100							
L. L.	23	M	0	4+	4+	2+	3.5×10^9*	95	B. S.	1½ hr.	M	0	0	0	0
							4.5×10^8‡	70							
J. F.	24	M	0	1+	3+	1+	6.4×10^9*	90	B. M.	2 hr.	M	0	0	0	0
							3.1×10^8§	80							

*Exchange transfusion.
†Subcutaneous injection.
‡Intramuscular injection.
§Subcutaneous and intramuscular injection.

TABLE IV. ATTEMPTED PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY USING BONE MARROW LEUKOCYTES AND CIRCULATING LEUKOCYTES INJECTED INTRAMUSCULARLY

DONOR	AGE	SEX	SENSITIVITY RESPONSES		TYPE OF CELL TRANSFERRED	ESTIMATED NO. OF CELLS TRANSFERRED	% VIABLE	RECIPIENT	AGE	SEX	PASSIVE TRANSFER RESPONSE	
			SK-SD	MUMPS							SK-SD	MUMPS
M. R.	30	M	4+	3+	White blood cells	1.3×10^9	99	B. R.	3 days	F	0	0
					Marrow cells	3.2×10^8	99					
M. M.	28	M	2+	2+	White blood cells	2.3×10^9	75	B. M.	4 hr.	M	0	0
					Marrow cells	2.2×10^8	95					
G. J.	30	M	4+	3+	White blood cells	2.1×10^9	90	B. J.	3 hr.	M	0	0
					Marrow cells	1.2×10^9	95					

TABLE V. SIMULTANEOUSLY ATTEMPTED PASSIVE TRANSFER OF DELAYED HYPERSENSITIVITY IN NEWBORN AND IMMUNOLOGICALLY MATURE INDIVIDUALS WITH VIABLE LEUKOCYTES OBTAINED FROM THE PERIPHERAL BLOOD

DONOR		SENSITIVITY RESPONSE				ESTIMATED NO. OF CELLS TRANSFERRED	% VIABLE	RECIPIENT		PASSIVE TRANSFER RESPONSE			
AGE	SEX	PPD	HISTOPLASMIN	TRICHOPHYTON	SK-SD			AGE	SEX	PPD	HISTOPLASMIN	TRICHOPHYTON	SK-SD
35	M	0	4+	0	4+	2.5×10^8	80	25	M	0	1+	0	3+*
						2.5×10^8	80	4 days	M	0	0	0	0
32	M	0	4+	0	5+	3.0×10^8	85	3 yrs.	F	0	3+	4+	0
						3.0×10^8	85	2 yrs.	F	0	4+	0	0
						3.0×10^8	85	19 days	F	0	0	0	0
25	M	0	3+	2+	4+	2.7×10^8	80	24 yrs.	M	0	5+	0	5+†
						2.7×10^8	80	9 mos.	F	0	0	0	2+
						2.7×10^8	80	10 days	F	0	0	0	0

*The recipient's pretransfer reaction was 3 plus. There was no change after the cell transfer.
†The recipient's pretransfer reaction was 2 plus. The reaction became more intense after the cell transfer.

delayed allergy in these recipients, whereas the leukocytes from the same donor, prepared in exactly the same fashion, induced delayed allergy in the nonsensitive, immunologically mature recipients in each instance.

DISCUSSION

These results establish, in contradistinction to the implications of Schlange's studies, that the newborn infant is a poor recipient of the passive transfer of delayed allergy as compared to the older child or adult. The failure of response of the newborn infant could reside at any of several levels of reaction. For example, it is possible that the newborn infant sustains passive transfer of delayed allergy, but that this is not readily revealed because of the relative resistance of the newborn infant to expression of inflammation in response to noxious stimuli. Eitzman and Smith[17] have shown that the nonspecific inflammatory response in the neonatal period is characterized by an inflammatory eosinophilia and a relative decrease in the mononuclear components, and, therefore, is qualitatively different from that of mature children. Although conflicting data are reported, some decrease in the phagocytic capacity and ameboid movement of neonatal leukocytes has been shown.[18, 19] The nonspecific failure of the neonatal skin to react to noxious stimuli, regardless of its mechanism, is, however, well established.[20]

Metaxas and Metaxas-Bühler[14] concluded that, since no latent period is required after passive transfer, the allergic reaction of the recipient must be in part or entirely a function of the transferred cells. If this conclusion is valid, our observation would indicate that the newborn infant is defective in marshalling the cellular and vascular elements which express delayed allergy. If, on the other hand, as Lawrence's studies[14] in man appear to indicate, the recipient plays an *active* role in the establishment and persistence of transferred delayed allergy, these observations may represent the disclosure of another aspect of the immaturity of the immune response of the newborn infant. Although these experiments support neither alternative, they demonstrate a deficiency in the newborn infant with respect to the ability to accept passively transferred delayed hypersensitivity. This deficiency may have important implications in terms of capacity of the newborn infant to defend himself against infection and various noxious substances, and it should be studied further.

SUMMARY

1. Attempts to transfer delayed allergy to newborn infants by injecting cells from donors of known sensitivity have been uniformly unsuccessful.

2. In separate and in simultaneous experiments, similar methods have regularly resulted in passive transfer of delayed allergy to immunologically mature children and adults.

3. The implications of these observations are discussed.

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LABORATORY METHODS

INFRARED ANALYSIS OF CARBON TETRACHLORIDE AND ETHANOL IN BLOOD

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This work was undertaken to determine the applicability of infrared spectroscopy to the quantitative analysis of carbon tetrachloride and/or ethanol in blood. A method for the extraction of chemicals from blood, and their subsequent quantitative analysis by an infrared technique, is described. Carbon tetrachloride-C^{14} was used to determine the efficiency of extraction of this chemical from the blood. It was found to be quantitative, within experimental error. When the highly water soluble ethanol was added to blood, only 5 per cent of the added amount was extracted. A correction factor was therefore used to calculate ethanol blood levels. Groups of 5 rabbits were exposed to carbon tetrachloride vapors, to ethanol via intubation, or to both chemicals simultaneously. Blood levels of carbon tetrachloride and ethanol were determined simultaneously by the method described. Blood levels of carbon tetrachloride were significantly higher, as were serum glutamic oxalacetic transaminase values, when the rabbits received both ethanol and carbon tetrachloride.

WHENEVER the question of acute or chronic poisoning from organic compounds arises, the physician immediately desires to know if a potentially toxic compound is actually circulating in the blood stream and what its concentration may be. In the field of toxicologic research there is also great need for rapid, simple methods for qualitative and quantitative analysis of organic compounds in tissues or biologic fluids of animals. Such methods can be used to correlate specific blood concentrations of toxic materials with tissue damage and physiologic effect. As an aid to environmental health studies, control of the workers' environment can also be accomplished on the basis of direct measurement of the compound in the blood or urine, as well as by monitoring it in the air. In a preliminary article[1] a simple, rapid, method which may be used for this type of analysis was outlined. The present report gives a more detailed description of this method, and demonstrates its application to a specific toxicologic problem.

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For the purpose of illustration and for the reasons outlined below, it was elected to measure the levels of carbon tetrachloride and ethanol in blood after separate and simultaneous exposure. (1) Carbon tetrachloride is almost insoluble in blood, whereas ethanol is readily soluble, therefore the simultaneous quantitative analysis of these two compounds illustrates the wide applicability of the method. (2) In addition, it was deemed desirable to observe what influence a high blood level of ethanol had upon the concentration of carbon tetrachloride in the blood of an animal exposed to carbon tetrachloride vapor. (3) Finally, it was desirable to correlate the results, if possible, with tissue damage as indicated by serum glutamic oxalacetic transaminase (SGOT) measurements.

MATERIALS AND METHODS

Method of Extraction and Analysis.—Five milliliters of oxalated blood is pipetted into a bacteriologic culture tube with an aluminum-lined screw cap. Ten milliliters of carbon disulfide (spectroscopic grade), or other suitable extracting solvent, is added and the tube is gently rocked through a 90 degree angle in an automatic rocker (or by hand) for 5 minutes. The rocking must be sufficiently gentle so that no stable emulsion is formed. The solvent layer (7 ml. can usually be recovered) is withdrawn by pipette and transferred to a standard infrared sample cell. A matching cell is filled with pure solvent and placed in the reference beam of the spectrometer to compensate for solvent absorptions. The spectrum is scanned in those regions where specific absorption bands of the chemical being determined are located.

The infrared cells used in this study had 16 mm. Teflon spacers, with small polyethylene spacers placed between the Teflon and the rock salt windows to prevent leakage. Although an improved optical null infrared spectrometer[2] was used in these experiments, most standard infrared spectrometers would probably give satisfactory results.

Since the strength of a specific absorption band for a given chemical is a simple function of the concentration of that chemical in the reference solvent, the concentration of the chemical is determined by comparison to known standards measured under identical conditions. Standards are prepared by diluting a weighed amount of the chemical to a known volume with the reference solvent. In order to relate the results from the infrared determination of a chemical in a solvent to the actual blood concentration, it is necessary to use a factor in the calculation. This factor is dependent upon the ratio of the volume of extracting solvent to the volume of blood extracted. In the studies reported, because the volume of carbon disulfide used was twice that of the blood, this factor was two. Therefore, the value for the blood concentration of the chemical, expressed as weight per unit volume, was obtained by multiplying the value found in the extracting solvent by two.

Efficiency of Extraction.—The efficiency of this method for the extraction of carbon tetrachloride and ethanol from blood was determined by adding a measured amount of each to blood and extracting as described above. No difficulty in obtaining a homogeneous mixture was encountered in adding a readily soluble compound such as ethanol to blood, but carbon tetrachloride, being almost insoluble, presented a formidable problem. It was found that it was impossible, even at these low concentrations, to add a weighed amount of carbon tetrachloride to blood and obtain an even distribution of this chemical throughout the sample. Therefore, C^{14}-labeled carbon tetrachloride was used for the preparation of the blood standard.

A weighed portion of carbon tetrachloride-C^{14}, with a specific activity of 2.82×10^4 DPM per milligram, was introduced below the surface of a 100 ml. sample of blood by means of 10 μl syringe. The vessel was stoppered and shaken gently. Approximately 6 ml. of blood was removed by pipette to a test tube. Five milliliters of this sample was

used for determination of carbon tetrachloride by the infrared technique and 0.1 ml. was used for C^{14} assay. The 0.1 ml. aliquot was shaken violently with Thixcin-toluene-scintillator gel to obtain uniform dispersion for counting. The 5 ml. aliquot was extracted with carbon disulfide, and two 0.1 ml. aliquots of the carbon disulfide solution were removed and added to vials containing toluene-scintillator solution for counting. The procedure was repeated on separate blood samples at two concentrations, and all analyses reported are the average of duplicate measurements at each step. Counting was carried out with a Tri-Carb* liquid scintillation spectrometer. Internal standard was added to each sample to determine the counting efficiency. The results, reported in parts per million, were calculated on the basis of the specific activity of the carbon tetrachloride-C^{14}.

Chemical Exposure of Rabbits.—The rabbits used in these experiments were healthy, young adults selected from the stock colony. Their diet and housing have been previously described.[3] The exposures to carbon tetrachloride were carried out in a 160 L., cubical, glass-walled chamber, also described previously.[4] The concentration of carbon tetrachloride in the chamber atmosphere was analyzed by direct combustion and was also monitored by a recording analyzer.[5] The carbon tetrachloride concentrations were within ± 10 per cent of the stated levels. Ethanol, 95 per cent, was administered intragastrically by means of a rubber catheter. All blood samples were obtained by cardiac puncture. The serum glutamic oxalacetic transaminase (SGOT) levels were determined by the Frankel method of Sigma Chemical Company.

The rabbits were divided into 5 groups and were treated as follows:

Two groups of 5 rabbits each, groups A and B, were exposed to carbon tetrachloride vapor, 5,000 ppm, for 4 hours. The rabbits in group B were fed 3 ml. per kilogram of ethanol immediately prior to the vapor exposure. Blood samples were taken for carbon tetrachloride and ethanol analysis 1½, 4, 4½, and 6½ hours after the onset of the vapor exposure.

Two groups of 6 rabbits each, groups C and D, were exposed to carbon tetrachloride vapor, 2,500 ppm, for 4 hours. The rabbits in group D were fed 3 ml. per kilogram of ethanol 1½ hours after the onset of the vapor exposure. Blood samples were taken for carbon tetrachloride, ethanol, and SGOT analysis 1½, 4, 4½, and 8½ hours after the onset of the vapor exposure.

Group E, comprised of 5 rabbits, served as ethanol controls for groups B and D. A blood sample for SGOT determination was obtained and 3 ml. per kilogram of ethanol was then administered. Blood samples for ethanol and SGOT analysis were taken 2½ and 7½ hours after ethanol administration.

RESULTS

An infrared spectrum of carbon disulfide, the solvent used for extraction in these studies, is shown in Fig. 1, *A*. When a matching 16 mm. cell filled with carbon disulfide was placed in the reference beam of the spectrometer, the common absorptions canceled and the base line shown in Fig. 1, *B* was obtained. In regions of high solvent absorption (e.g., 6 to 7.4 μ) the pen servo becomes "dead" and a downward drift is observed in the differential spectrum. Any carbon disulfide soluble organic compound having absorption bands in regions where carbon disulfide transmits some of the incident radiation may be identified both qualitatively and quantitatively. The spectra of carbon tetrachloride and ethanol in carbon disulfide are shown in Fig. 2, *A* and *B*, respectively. The strong absorption of carbon tetrachloride at 12.8 μ made it possible to detect concentrations as low as 1 ppm. in carbon

*Packard Instrument Company, LaGrange, Ill.

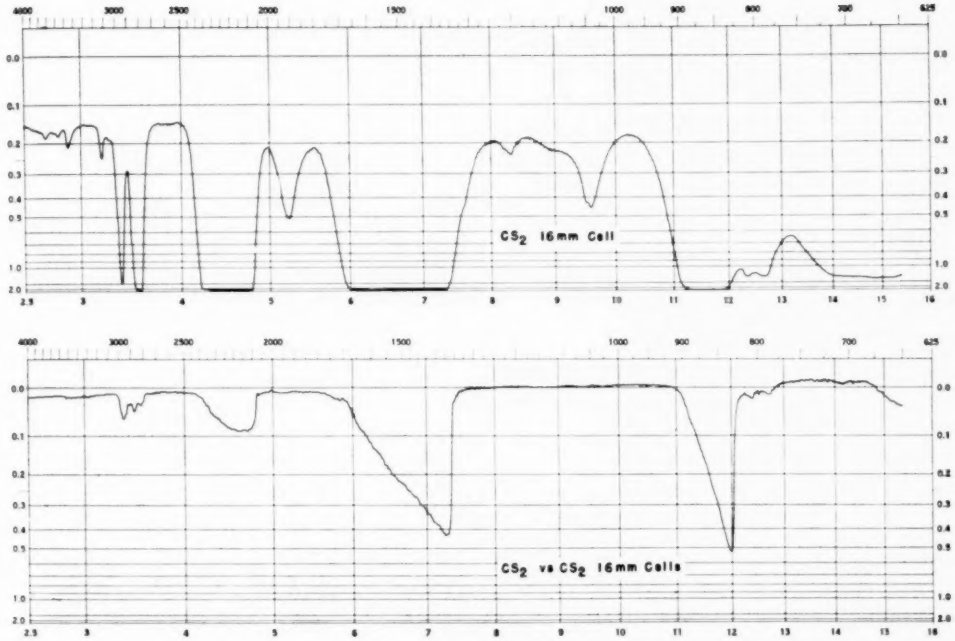

Fig. 1.—*A*, Infrared spectrum of carbon disulfide (16 mm. cell). *B*, Differential scan of carbon disulfide in matching 16 mm. absorption cells.

disulfide solution. Ethanol has a relatively weaker absorption at 9.55 μ so that the lower limit of sensitivity to this compound in carbon disulfide was roughly 10 ppm.

Table I presents the data of the extraction of carbon tetrachloride and ethanol from the prepared blood standards. From the radiotracer data it is evident that all of the carbon tetrachloride present in the aliquot of blood was effectively extracted by the carbon disulfide. The discrepancy between the weighed amount of carbon tetrachloride added to the blood standard and the amount actually present in the aliquot removed as measured by the radiotracer method illustrates the difficulty of obtaining an even distribution of carbon tetrachloride in blood. Although no attempt was made to locate the remaining carbon tetrachloride, it was assumed to have evaporated or settled to the bottom of the flask. It was found necessary to remove aliquots for radiotracer analysis and solvent extraction almost simultaneously, and from a relatively small volume of blood standard, in order to obtain close agreement between these two methods of analysis. The infrared analysis of the blood standards for ethanol shows that only 4.5 to 4.9 per cent of this compound was extracted. In order to obtain the reported blood concentration of ethanol in the rabbits from groups B, D, and E, the observed blood concentration was multiplied by a factor of 1/0.047. This low efficiency of extraction is related to the greater solubility of ethanol in blood than in carbon disulfide. It was assumed that all of the ethanol added to the blood standard was evenly distributed because of its high solubility in blood, and because of

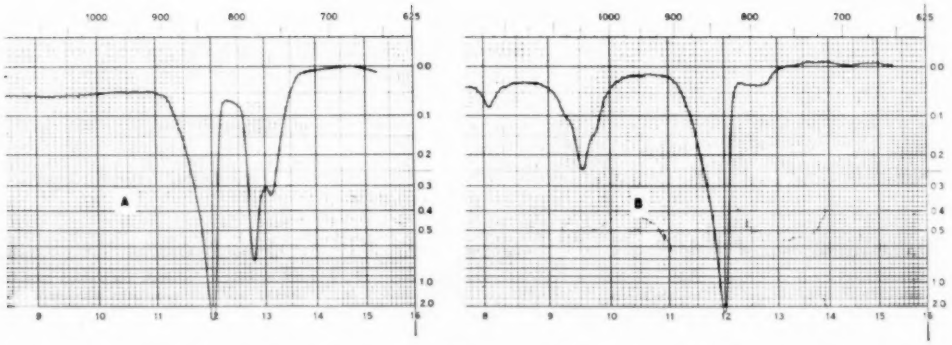

Fig. 2.—*A*, Infrared spectrum of carbon tetrachloride, 100 ppm, in carbon disulfide. *B*, Infrared spectrum of ethanol, 100 ppm, in carbon disulfide.

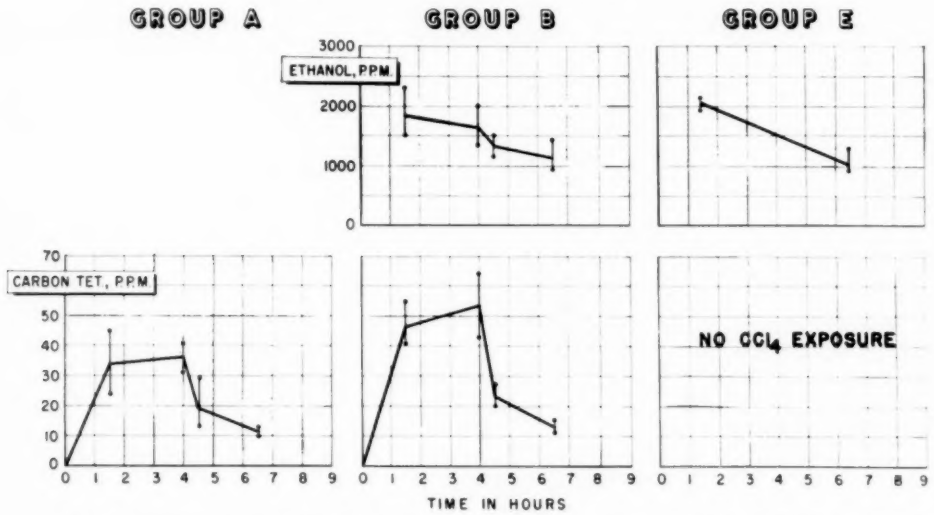

Fig. 3.—Blood concentrations of carbon tetrachloride and/or ethanol in rabbits of Groups A, B, and E. *Group A*, Carbon tetrachloride, 5,000 ppm. *Group B*, Carbon tetrachloride, 5,000 ppm, plus ethanol, 3 ml. per kilogram. *Group E*, Ethanol, 3 ml. per kilogram.

the reproducibility of extraction from several standards. The use of methylene chloride as an extracting solvent improved the extraction efficiency considerably; however, this solvent absorbed radiation at 12.8 μ and prevented the simultaneous analysis of carbon tetrachloride.

Fig. 3 shows the average, together with the maximum and minimum concentrations of carbon tetrachloride and ethanol in the blood of rabbits from groups A, B, and E. It is interesting to note that the blood concentrations of carbon tetrachloride were significantly higher in group B, the group of rabbits given ethanol prior to the exposure. This elevation was only slightly significant after 1½ hours of exposure ($p=0.03$) but after 4 hours was quite significant ($p=0.013$).

Fig. 4 shows similar values for blood concentrations and the SGOT levels of groups C, D, and E. During and immediately after the exposure

the average blood concentrations of carbon tetrachloride in groups C and D were approximately one half those found for groups A and B, respectively, which indicates that carbon tetrachloride uptake was almost directly proportional to the vapor concentration at the two levels studied. In this experiment, group D (those rabbits receiving ethanol) was found to have an only slightly higher average blood concentration of carbon tetrachloride at the conclusion of the exposure than had group C (those rabbits receiving no ethanol). A comparison of the average SGOT levels of these groups also indicates the combined effect of the two chemicals. Although the average levels were essentially the same at 1½ hours, the average level for group D rose to over twice that of group C after 4 hours ($p=0.008$). However, this great difference in levels was not maintained throughout the remainder of the experiment. After 8½ hours the differences were of questionable significance ($p=0.05$). The results of the administration of ethanol to group E show that this compound did not by itself cause a rise in the SGOT level. It is also evident that the cardiac punctures did not raise the SGOT levels.

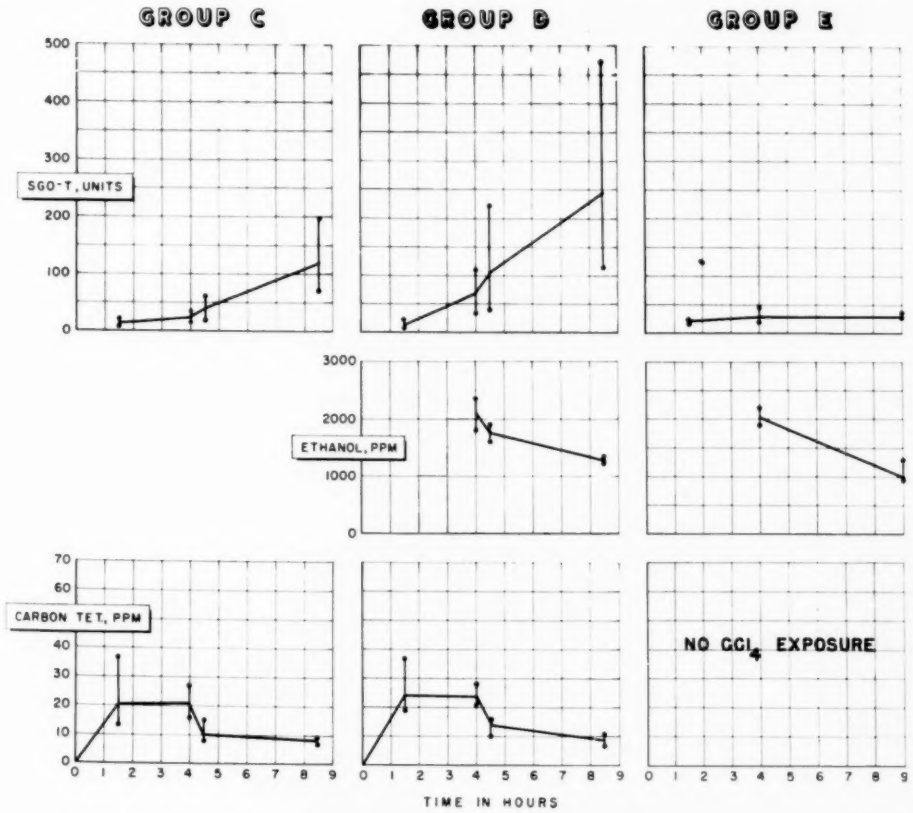

Fig. 4.—SGOT levels and blood concentrations of carbon tetrachloride and/or ethanol in rabbits of Groups C, D, and E. *Group C*, Carbon tetrachloride, 2,500 ppm. *Group D*, Carbon tetrachloride, 2,500 ppm, plus ethanol, 3 ml. per kilogram. *Group E*, Ethanol, 3 ml. per kilogram.

and that though ethanol either directly or indirectly caused a rise in average carbon tetrachloride blood concentration, the exposure to carbon tetrachloride had no effect upon the average ethanol blood concentration.

TABLE I. CARBON DISULFIDE EXTRACTION OF CARBON TETRACHLORIDE AND ETHANOL FROM BLOOD STANDARDS

CHEMICAL	ADDED TO BLOOD (PPM)	FOUND IN BLOOD SAMPLE BY TRACER METHOD (PPM)	EXTRACTED FROM BLOOD SAMPLE WITH CS_2	
			INFRARED METHOD (PPM)	TRACER METHOD (PPM)
CCl_4	116	38	40	39
	116	31	25	27
	120	25	22	23
	120	26	30	28
Ethanol	3,020		148	
			144	
	1,560		74	
			78	

DISCUSSION

The infrared solvent extraction method presented offers a sensitive, rapid, and simple technique for the analysis of organic compounds in blood. Generally, infrared has not been considered to have sensitivity in the parts per million range, except in certain specialized cases. However, to achieve sensitivity in this range, it is only necessary to use longer cells. This means more solvent absorption, but this can be compensated provided a small per cent of the total energy applied is transmitted in the region of interest. The data presented were obtained with the use of a 16 mm. cell. Longer cells would increase the sensitivity, but would require a larger sample, and, in addition, would give rise to more solvent absorption. Sensitivity may perhaps be more easily increased by extracting a larger volume of blood with the same ratio of solvent to blood, and subsequently concentrating the chemical in the extractant by evaporation, or by successive extractions in cases where the compound is not very soluble in the extractant.

Because each organic compound has a specific infrared absorption spectrum, the method presented generally enables definite identification of the compound or compounds being determined. Because of the unusually strong solvent absorptions encountered when very thick cells are used, it may be necessary to try several solvents before the entire sample spectrum is revealed. Carbon disulfide is an excellent solvent for survey work because large portions of its spectrum have low absorption. The toxic and explosion hazards of this material, though reduced when used with care in a good hood, may outweigh the superiority of its infrared transparency. Other solvents which have been useful in certain specific applications are methylene chloride, carbon tetrachloride, and bromoform. The choice of solvent will largely be determined by its infrared transparency at wavelengths where the sample has strong absorption bands and at cell thicknesses giving the required sensitivity. Extraction efficiency, toxicity, economy, etc., are important but secondary factors in this choice.

Although the main purpose of this article is to present an analytic method, the data obtained from the illustrative experiment are of interest and deserve comment. It has been a well-accepted clinical observation that the ingestion of alcohol at or near time of exposure to carbon tetrachloride greatly predisposes the patient to the serious effects of the chemical, and there has been speculation as to the mechanism involved.[6, 7] The rise of the SGOT in the rabbits receiving both ethanol and carbon tetrachloride to levels twice that of rabbits receiving only carbon tetrachloride suggests that the combination of ethanol and carbon tetrachloride is more rapidly injurious to the animal than is either chemical alone. Although the number of animals in each series was small, those rabbits which received the combination of chemicals evidenced higher blood concentration of carbon tetrachloride than did those rabbits exposed only to carbon tetrachloride. It is not known if this effect of ethanol upon the blood concentration of carbon tetrachloride is direct or if it is rather an indirect effect, possibly mediated by serum lipid concentrations.

In addition to the type of study described to illustrate this method for the determination of chemical levels in blood, this method should also be applicable to the determination of concentrations of medications and their metabolites in blood, urine and other biologic fluids and tissues. Many medications are more soluble in organic solvents than in blood and water, and, therefore, ideally lend themselves to analysis by this method. Abnormal concentrations of metabolites may also be detected by this method. For example, analysis of acetone in blood[1] is easily accomplished by extraction with either carbon disulfide, carbon tetrachloride, or methylene chloride. It is felt that this method, though simple and rapid, has a wide range of applicability in almost all fields of biologic research.

SUMMARY

A simple, rapid method for the simultaneous identification and assay of organic chemicals in the blood has been demonstrated in the analysis of carbon tetrachloride and ethanol. The method was shown to be made quantitative for carbon tetrachloride by the use of C^{14}-labeled material.

A significant increase in the level of carbon tetrachloride circulating in the blood was found when rabbits were exposed simultaneously to high doses of carbon tetrachloride and ethanol. An increase in the serum glutamic oxalacetic transaminase levels was also observed.

This method appears applicable to the assay of chemicals, medications, and metabolites in many biologic fluids and tissues.

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COLORIMETRIC METHOD FOR DETERMINATION OF ERYTHROCYTE GLUTATHIONE

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A colorimetric method for the determination of erythrocyte glutathione (GSH) is described. The method is based upon the reaction of an aromatic disulfide, bis(p-nitrophenyl) disulfide, with mercaptans with the release of the yellow colored nitrobenzenethiol anion. The color which develops is stable.

The glutathione stability test has been performed utilizing this method and the results compare favorably with those obtained using the nitroprusside method of Grunert and Phillips.[3] The simplicity of the procedure and the stability of the color reaction make the method readily adaptable by the routine laboratory for the performance of the glutathione stability test.

THE DEVELOPMENT of the glutathione stability test by Beutler[1] has provided a method for the detection of individuals whose erythrocytes are susceptible to hemolysis by certain drugs, e.g., primaquine. A positive glutathione stability test is characterized by a marked decrease in the reduced glutathione content of erythrocytes after the incubation of blood with acetylphenylhydrazine, whereas in nonsensitive erythrocytes the glutathione content is but slightly decreased or unchanged. The fundamental defect in sensitive erythrocytes is a genetically determined deficiency of the enzyme, glucose-6-phosphate dehydrogenase, and the alteration in glutathione content is an indirect measure of the enzyme abnormality.[2]

The nitroprusside method as modified by Grunert and Phillips[3] has been utilized in the glutathione stability test described by Beutler. This method is based on the color which develops when SH groups react with nitroprusside but is not specific for mercaptans. The method is somewhat time consuming, and the color which develops is unstable. Ellman[4] has described a method for the determination of low concentrations of SH groups based on the reaction of an aromatic disulfide [bis(p-nitrophenyl) disulfide] with mercaptans with the release of the yellow colored nitrobenzenethiol anion. The color of the nitrobenzenethiol anion is stable. We have adapted this method for the determination of erythrocyte glutathione in the glutathione stability test. The results obtained by this method and a comparison by the nitroprusside method constitutes the present report.

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MATERIAL AND METHODS

1. Bis(p-nitrophenyl) disulfide (PNPD).* The PNPD should be recrystallized from glacial acetic acid, melting point 183 to 184° C. Glacial acetic acid was added to PNPD and the solution was boiled for 15 minutes. The solution was allowed to cool; the glacial acetic acid was decanted. The crystals of PNPD were placed on a watch glass, evaporated for 12 hours, and dried in an oven at 105° C. The PNPD reagent is prepared by dissolving 30.8 mg. of PNPD in 100 ml. of acetone. The reagent is stable for 6 to 8 weeks when stored in plastic reagent bottles in the dark.

2. Phosphate buffer, 0.15 M, pH 8.4, is used.

3. Acetyl phenylhydrazine, 10 per cent solution in methyl alcohol, is used.

PROCEDURE

The blood specimens analyzed were collected in acid citrate dextrose solution B. Blood proteins were precipitated by the Folin-Wu method.[5] Two milliliters of whole blood was pipetted slowly into a 50 ml. Erlenmeyer flask which contained 16 ml. of 1/12 normal sulfuric acid. After the addition of sulfuric acid, the specimen was allowed to stand for 10 minutes to assure complete hemolysis of the erythrocytes and then 2 ml. of 10 per cent sodium tungstate was added with constant agitation to obtain a fine precipitate and the mixture was filtered through Whatman No. 1 filter paper.

The stepwise procedure following the preparation of filtrate is: (1) 4 ml. of the filtrate is pipetted into a 50 ml. Erlenmeyer flask; (2) 1 ml. of the phosphate buffer, pH 8.4 is added to the filtrate; (3) 4 ml. of acetone is added; and (4) 1 ml. of the PNPD reagent is added.

A deep yellow color develops promptly after the addition of the PNPD reagent. The specimens were read in a Coleman Junior spectrophotometer at a wavelength of 412 mμ.

A reagent blank was prepared by substituting 4 ml. of distilled water for the filtrate in the procedure. The blank should be read promptly since PNPD is slowly hydrolyzed with the spontaneous development of color in the blank.

A reference standard was prepared daily from an aqueous solution which contained 0.4 mg. glutathione per milliliter. The glutathione standard was found to be stable for 5 to 7 days when stored in the refrigerator.

The effect of acetylphenylhydrazine on erythrocyte glutathione content was determined by incubating 2 ml. of whole blood with 10 mg. of acetylphenylhydrazine for 2 hours at 37° C. in a water bath. The tubes (200 by 25 mm.) which contained 10 mg. of dried acetylphenylhydrazine were prepared as described by Beutler.[1] Proteins were precipitated by the addition of sulfuric acid and sodium tungstate to the incubation tubes.

In order to determine the accuracy of the method, the recovery of added glutathione was determined daily by adding glutathione to whole blood specimens after the addition of the sulfuric acid and prior to the addition of the sodium tungstate. The percentage of recovery ranged from 90 to 97 per cent and averaged 94 per cent for all determinations.

The following precautions have been found necessary to produce 90+ per cent recovery of added glutathione and consistent results.

1. The results obtained on blood specimens with a hematocrit of less than 20 per cent are not reliable and the hematocrit should be adjusted to 20 per cent or preferably to 30 to 40 per cent when the glutathione stability test is performed by this method.

2. The pH of the filtrate after the addition of the buffer should be greater than 7.8 in order to insure maximum color development, since p-nitrobenzenethiol is an acid with a pK of 5.1 and at a pH greater than 7.8 is 99 per cent ionized. The intensity of the color development is dependent upon the concentration of the nitrobenzenethiol anion. The pH of the buffer should be checked frequently to determine its stability.

*Eastman Organic Chemicals T 1855.

3. Glutathione undergoes auto-oxidation at the pH of the filtrate obtained utilizing the Folin-Wu method for precipitation of the plasma proteins. The rate of auto-oxidation is negligible for the first 90 minutes after preparation of the filtrate but more rapid thereafter. Auto-oxidation is accelerated by heat and may be retarded by maintaining the temperature of the filtrate at 4° C. in ice water.

The concentration of glutathione expressed as milligrams per 100 ml. of red cells was determined according to the following formula:

$$\frac{\frac{\text{O.D.u}}{\text{O.D.s}} \times \text{C Sta} \times \text{d}}{\text{HCT}} \times 100 = \text{mg. GSH/100 ml. R.B.C.}$$

O.D.u = optical density unknown. O.D.s = optical density standard. C Sta = concentration of standard. d = dilution of filtrate (2.5).

RESULTS

The results of the glutathione stability test performed in 58 Negro females, 38 Negro males, 18 Caucasian females, and 21 Caucasian males are shown in Table I. The range of glutathione concentration and the percentage of sensitive individuals in the various racial groups are in essential agreement with the results obtained by the nitroprusside method by various investigators.

TABLE I. GLUTATHIONE CONTENT OF ERYTHROCYTES BY PNPD METHOD

RACE	SEX	NO. OF PATIENTS	RANGE (MG./100 ML.)	AVERAGE	% SENSITIVE < 20 MG./100 ML.)
Negro	M	38	42-110	58	4.5
Negro	F	58	47-86	63	8.1
White	M	21	51-80	64	0
White	F	18	44-90	69	0

Table II shows the results of the glutathione stability test on the same blood samples by the method described here compared with the Grunert and Phillips method.[3]

TABLE II

PATIENT	RACE	SEX	NITROPRUSSIDE		PNPD	
			PRE-INCUBATED	POST-INCUBATED	PRE-INCUBATED	POST-INCUBATED
A. L.	Negro	M	63.5	58.8	61.2	60.5
H. F.	Negro	M	58.6	45.5	65.0	61.2
H. M.	Negro	M	52.0	17.8	52.6	10.8
G. K.	Negro	M	39.0	31.8	40.5	33.6
J. M.	Negro	F	52.0	45.0	48.5	43.5
R. T.	Negro	F	43.0	47.5	58.0	53.2
A. Y.	Negro	F	60.5	56.5	60.5	57.2
B. R.	Negro	F	52.0	35.5	47.0	35.0
S. P.	Negro	F	53.8	34.2	51.0	37.6
G. W.	White	M	63.2	63.2	63.0	53.0
J. M.	White	M	66.0	51.2	57.9	54.8
J. P.	White	M	47.0	43.5	53.0	48.2
J. K.	White	M	61.8	52.0	68.4	57.6
J. B.	White	M	61.2	56.2	60.8	56.2
L. H.	White	F	57.2	50.4	60.2	53.8
V. P.	White	F	62.0	64.4	63.0	55.2
R. W.	White	F	40.2	40.2	48.5	45.5
M. R.	White	F	72.2	66.0	68.0	64.0

DISCUSSION

The PNPD method has proved to be a rapid, reliable, and accurate method for determining the glutathione concentration of red cells and is applicable for use in the glutathione stability test. The determination of glutathione by biochemical means is difficult because of the tendency of the glutathione to undergo auto-oxidation. Auto-oxidation is more apt to occur at the pH of filtrate prepared by the Folin-Wu method; however, repeated determinations have shown that this is not a significant factor if the filtrates are processed promptly after preparation. The glutathione content determined immediately at 30, 60, and 90 minutes after preparation of the filtrates showed a variation of not greater than 1 mg. per cent, but auto-oxidation occurred if the filtrates were allowed to stand longer. It is not possible to store the filtrates for later determinations.

Ergothioneine does not give a color reaction with nitroprusside but does with PNPD. Despite the fact that PNPD produces a color reaction with aqueous solutions of ergothioneine, it has not been possible to recover added ergothioneine from blood samples. The failure to recover added ergothioneine from blood samples and the close correlation with the nitroprusside method implies that red cell ergothioneine is not measured by the procedure as described.

The method is simple, reliable, and is readily adapted for use in the routine laboratory for the determination of the glutathione stability test.

SUMMARY

A colorimetric method for the determination of low concentrations of SH groups, based on the reaction of mercaptans with an aromatic disulfide, has been adapted for use in the glutathione stability test. The method as modified for determination of erythrocyte glutathione and a comparison with the nitroprusside method are described.

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ENZYMATIC ESTIMATION OF ERYTHROCYTIC GALACTOSE-1-PHOSPHATE

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A modification is presented of an erythrocytic assay which was published earlier[8] and which has proved useful in the diagnosis of galactosemia. The modification permits estimation of the concentration of galactose-1-phosphate in the erythrocytes of individuals with galactosemia. It has been previously recognized that the successful clinical management of patients with galactosemia depends upon the exclusion of galactose from their diet. The concentration of erythrocytic galactose-1-phosphate is a sensitive indication of exposure of such individuals to galactose. The technique of the assay is described and evidence is presented that the assay may be used to monitor the degree of dietary control.

INFANTS with galactosemia usually develop jaundice, hepatic damage, growth failure, and cataracts if they are allowed to take the customary feedings of breast or cow's milk.[1-3] In the early stages these complications are reversible and preventable if galactose containing foods, principally milk, are excluded from the diet. Of current interest is the degree to which such dietary measure may also prevent the mental deficiency that often accompanies galactosemia.[3] Strict maintenance of a galactose-free diet is made difficult by the clandestine eating habits of children and by the frequency with which milk is an unmentioned ingredient of many modern foods. Opinions differ[4] as to whether or not some milk substitutes are sufficiently free of galactose to permit their use in galactosemia. A sensitive method for monitoring the adequacy of the diet is, therefore, needed. Urinary and blood galactose levels are variable and may be normal at the time of examination of the symptomatic child. In the galactosemic individual, erythrocytic galactose-1-phosphate* is cumulative, however, and tends to persist for many days or weeks after extensive ingestion of milk.[5] Furthermore, there is evidence[3, 6, 7] that the toxicity of galactose in galactosemia is related to the intracellular Gal-1-P. Measurement of erythrocytic Gal-1-P therefore seems a more sensitive and appropriate index of the extent to which the patient is under dietary control than does the measurement of urinary or blood galactose.

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*The following abbreviations are used: Gal-1-P for α-D-galactose-1-phosphate, UDPG for uridine diphosphoglucose, UDPGal for uridine diphosphogalactose, DPN for diphosphopyridine nucleotide, DPNH for reduced diphosphopyridine nucleotide, and G-1-P for α-D-glucose-1-phosphate.

Erythrocytic Gal-1-P can be estimated by a modification of the specific enzymatic assay used for the diagnosis of galactosemia and reported earlier in this JOURNAL.[8] The earlier assay has as its purpose the detection of the enzyme Gal-1-P uridyl transferase in hemolyzates of human erythrocytes. The enzyme catalyzes the reaction:

$$\text{Gal-1-P} + \text{UDPG} \rightleftharpoons \text{G-1-P} + \text{UDPGal} \quad (1)$$

and is inactive or absent in galactosemic persons. The enzyme is detected in the earlier assay by incubating the suspected hemolyzate with Gal-1-P and UDPG. At the end of the incubation the mixtures are deproteinized and the amount of UDPG which remains is determined by using as a reagent the enzyme UDPG dehydrogenase, which specifically oxidizes UDPG to UDP-glucuronic acid in the presence of DPN:

$$\text{UDPG} + 2\ \text{DPN} \rightarrow \text{UDP-glucuronic acid} + 2\ \text{DPNH}. \quad (2)$$

The amount of DPNH formed, as measured by optical density (O.D.) at 340 mμ wavelength of light, is proportional to the amount of UDPG which remains after incubation.

The assay to be described has as its purpose the measurement of the amount of Gal-1-P in galactosemic hemolyzate. The presence of Gal-1-P uridyl transferase is ensured by the addition of normal hemolyzate. Purified Gal-1-P (Reaction 1) is omitted. The unknown becomes the amount of Gal-1-P intrinsic to the galactosemic hemolyzate. Sufficient normal hemolyzate and incubation time are used to bring Reaction (1) to equilibrium. The amount of UDPG consumed becomes a measure of the amount of Gal-1-P present.

MATERIALS AND METHODS

The equipment and the type, concentration, and method of preparation of reagents are the same as specified in the UDPG consumption test.[8] The following procedural modifications are necessary for the estimation of Gal-1-P.

Preparation of Blood Sample.—Both heparinized galactosemic and heparinized, fresh, normal blood are used. After preparation of the saline-washed, packed, erythrocytes in the manner previously described,[8] the cells are hemolyzed by rapidly freezing and thawing 3 times. This is done by placing the tubes of packed cells alternately in a beaker of acetone with dry ice and in a beaker of water at room temperature. In contrast to the earlier assay, the packed cell galactosemic hemolyzate is not diluted with water and no attempt is made to remove stroma from either hemolyzate. The hemolyzates of normal and galactosemic blood are preincubated in a 37° C. bath for 5 and 10 minutes, respectively, before being used.*

Incubation.—To each of three 12 ml. heavy-duty Pyrex centrifuge tubes is added exactly 300 μl of the same Tris buffer-UDPG solution as is used in the previous assay.[8] It is recommended that the same 300 μl micropipette be used for all 3 tubes. To the first and second tube is added 100 μl of packed-cell, galactosemic hemolyzate. One hundred microliters of water is added to the third tube. The packed-cell, normal hemolyzate is mixed with

*Erythrocytes of normal persons are able to metabolize galactose, yet one of the enzymes necessary for the utilization of galactose, UDPGal-4-epimerase, has been found not to function in hemolyzates unless DPN is added. This aberration[9] has been explained by the finding that supplementary DPN is unnecessary if nicotinamide is added during hemolysis. DPN is required if nicotinamide is not added until sometime after hemolysis. A nicotinamide-inhibitable DPNase apparently destroys intrinsic DPN upon hemolysis. Since the UDPGal-4-epimerase must be inactive for the estimation of Gal-1-P, hemolyzates are preincubated at 37° C. in order to insure removal of DPN.

an equal volume of distilled water in order that it may be more easily pipetted and in order that the incubation mixture may be brought to the desired volume. Two hundred microliters of this diluted normal hemolyzate is added to all 3 tubes. The third tube should be prepared in duplicate if it has not been replicated in the course of assaying other samples of blood on the same day. The contents of each tube are gently stirred with the tip of a micropipette. The 3 tubes are incubated for 1 hour in a 37° C. water bath.

Determination of UDPG.—The deproteinization of the incubation mixtures and the assay for residual UDPG are as described in the previous assay. To increase the accuracy of estimation of UDPG two readings are made at both 340 and 400 mμ on each of the 3 cuvettes before the UDPG dehydrogenase is added as the last item in the cuvettes. The readings at 400 mμ reveal changes in turbidity and nonspecific absorption. The same amount of UDPG dehydrogenase is delivered by micropipette into all cuvettes and the cuvettes are remixed by inverting, as described.[8] Readings are taken at 340 mμ promptly and at intervals of several minutes until the readings reach a definite maximum. Sufficient UDPG dehydrogenase should be used to give a maximum within 30 minutes after the reaction is started. Readings are taken at 400 mμ occasionally and when the maximum is reached. The value $(O.D._{340} - O.D._{400})$ obtained before the addition of UDPG dehydrogenase is subtracted from the maximum $(O.D._{340} - O.D._{400})$ obtained thereafter in order to obtain a value which is designated ΔD_{340} and which is proportional to the amount of UDPG in the cuvette. After reaching a maximum, the $(O.D._{340} - O.D._{400})$ value often diminishes slowly. The ΔD_{340} for the third cuvette is usually 0.30 to 0.36.

Calculation of Gal-1-P.—The average ΔD_{340} for the first 2 cuvettes (duplicates) is subtracted from the ΔD_{340} of the third cuvette (blank) to give a value which is designated $\Delta\Delta D_{340}$ and which is proportional to the amount of UDPGal or Gal-1-P in the first 2 tubes. The $\Delta\Delta D_{340}$ is multiplied by 115 to give the concentration of Gal-1-P (free ester) in milligrams per 100 ml. of the packed erythrocyte preparation from the galactosemic individual. With hematocrits above 90 per cent, correction for incomplete packing of the erythrocytes is beyond the accuracy of the method and is unnecessary.

Hemolyzates, deproteinized extracts, and all reagents are kept at 0° C. when not being read in the spectrophotometer or incubated. Care is taken that droplets of water do not dilute the preparations as they are moved in and out of the water or ice baths.

Verification of the Method.—One worker added measured increments of a Gal-1-P solution to 11 pairs of duplicate tubes which contained a small amount of galactosemic hemolyzate which was low in intrinsic Gal-1-P. Each tube was given a code number and the concentration of Gal-1-P in each tube was determined by another worker in the manner described, except that each tube was not further duplicated. Tubes which resulted in more than one third consumption of the UDPG were reanalyzed, using one half or one fourth the usual amount of galactosemic hemolyzate and correcting the deficit in volume with distilled water. The Gal-1-P solution was standardized by hydrolyzing for 20 minutes at 100° C. in 0.02M HCl at concentrations of approximately 1 μM Gal-1-P per milliliter of diluted acid. Reducing substance was then determined by the method of Park and Johnson,[10] using recrystallized galactose as a standard. The Gal-1-P concentration of the standard was then computed on the assumption that all reducing substance of the hydrolyzate represented α-D galactose-1-phosphate. Paper chromatography of the unhydrolyzed and hydrolyzed Gal-1-P preparation was carried out using a system[11] of ethyl acetate, pyridine, and water, with running times of 5 and 16 hours. The chromatograph was developed with silver nitrate.[12]

RESULTS AND DISCUSSION

Accuracy of the Method. Replicates of the third tube had ΔD_{340} values which agreed with one another within ±0.01 O.D. units, or approximately 3 to 5 per cent of the total. Under the conditions stated, up to 35 mg. Gal-1-P per 100 ml. (Fig. 1) can be measured, and average values have errors of ±1.7 mg. per 100 ml. or less. Paper chromatography of the hydrolyzed and

unhydrolyzed Gal-1-P standard revealed 10 to 20 per cent of the reducing substance to be from substances other than Gal-1-P. Although quantitative estimation of Gal-1-P values above 10 mg. per 100 ml. is clinically of less importance, the amount of galactosemic hemolyzate should be halved or quartered if accuracy in this range is desired. Equilibrium conditions are such as to lead to loss of linearity if more than one half of the UDPG is consumed. When the dietary history suggests that the level of Gal-1-P may be low, this level can be determined with greater accuracy if the concentration of UDPG in the incubation mixture is decreased by one half and if twice as much deproteinized extract is added to the cuvettes. A corresponding volume of water is then omitted from the cuvettes in order to maintain the same total volume.* Accuracy is dependent upon careful micropipetting technique, procuring deproteinized extracts which are relatively free of particles, and using UDPG dehydrogenase solutions with minimum turbidity, i.e., less than 0.03 O.D. increase at 400 mμ when added to the assay mixture.

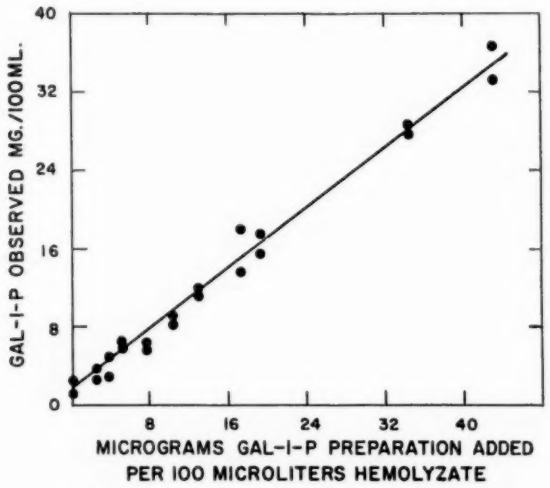

Fig. 1.—Determination of Gal-1-P in galactosemic hemolyzate which contained added Gal-1-P.

As in the UDPG consumption test, the ΔD_{340} can be further checked by use of UDPGal-4-epimerase.[13] Six additional unknowns determined in this manner, using UDPGal-4-epimerase purified from galactose adapted yeast,[14] gave results comparable to those shown in Fig. 1.

Study of the Hemolyzates of Galactosemic Children.—The Gal-1-P was estimated (Table I) in the bloods of galactosemic children who were known by clinical signs to have galactosemia and who received varying degrees of

*As determined from 17 assays utilizing 100μl of galactosemic hemolyzate and 300μl of deproteinized extract, the standard deviation of the method was 0.67 mg. per 100 ml. A limit of accuracy for the method was arbitrarily taken as 2.5 times this value or 1.7 mg. per 100 ml. In our experience, this error is predominantly caused by ±0.010 O.D. unit of variation which occurs in the reading of the cuvettes independently of the dilutions used. Accordingly, the limits of accuracy are probably ±3.4 and ±0.8 mg. per 100 ml. for the modifications for the high and low range of Gal-1-P, respectively.

dietary control.* The status of all subjects listed in Table I had been confirmed by the UDPG consumption test. The results are essentially in agreement with those of Schwarz and associates,[15, 16] who found < 1, < 2, and 9 to over 20 mg. per 100 ml. for the erythrocytic Gal-1-P of normal persons, galactosemic children on a milk-free diet, and galactosemic children taking milk, respectively. Although the method by Schwarz and co-workers[5, 16] of estimating Gal-1-P requires no special reagents and permits measurements in the low range, i.e., less than 2 mg. Gal-1-P per 100 ml., measurement of Gal-1-P by that method requires 2 to 3 days to complete and necessitates quantitating the amount of Gal-1-P by visual estimation of the intensity of a chromatographic spot. The enzymatic estimation of Gal-1-P has the advantage that it can be made within one afternoon and with the same reagents

TABLE I. ERYTHROCYTIC GALACTOSE-1-PHOSPHATE CONCENTRATIONS IN VARIOUS PERSONS

SUBJECTS	ERYTHROCYTIC GAL-1-P (MG./100 ML.*)
Galactosemic Subjects.—	
A: boy, 3½ mo. old, on a milk-free diet except for receiving milk for 24 hours prior to test.	19.8 ± 3.4
A: 1 yr. later, on milk-free diet.	0.7 ± 0.8
B: boy, 11 yr. old, on milk-poor diet, 32 hr. after 40 Gm. galactose.	6.5 ± 1.7
B: 2 wk. later, on milk-free diet.	2.0 ± 1.7
C: girl, 16 mo. old, on milk-free diet.	1.1 ± 0.8
D: boy, 5 yr. old, on milk-free diet.	0.0 ± 1.7
Nongalactosemic Subjects.—	
E: girl, 2½ yr. old, mentally defective.	0.0 ± 0.8
F: Normal newborn girl, cord blood.	1.7 ± 0.8
G: man, 31 yr. old, normal.	0.0 ± 0.8

*Plus or minus (±) limit of accuracy (2.5 × estimated standard deviation of the method) corresponding to dilution used.

and stock solutions that are used in the UDPG consumption test[8] for the diagnosis of galactosemia. By the enzymatic estimation, values of Gal-1-P below 2.8 mg. per 100 ml. cannot at the present time be considered significantly elevated for the galactosemic child. However, the "safe" level of erythrocytic Gal-1-P has not yet been determined, and the possibility must not be ignored that future studies may place this level below any presently existing arbitrary value.

SUMMARY

The uridine diphosphoglucose (UDPG) consumption test, used in the diagnosing of galactosemia, can be modified to permit an estimate of erythrocytic galactose-1-phosphate and to detect certain errors in the diet of the individual with galactosemia.

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